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INDICATIONS RESPECTING THE NATURE OF THE INFECTIVE PARTICLES IN THE MOSAIC DISEASE OF TOBACCO[1]

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Among plant pathologists there is to-day no topic of more engaging interest and no problem more difficult than that of the nature of the causal agency in mosaic and allied plant diseases. Possibly we might extend this statement so as to comprehend at the same time the causal factors involved in those types of vegetative variegation, or modified pigmentation, whether infectious or not, which afford the decorative mottled, spotted, and striped plants so much cultivated for foliage effects.

At various times almost every conceivable view has been held as to the nature of the etiological agent in tobacco mosaic, but from the earliest experiments it has been perfectly clear that the disease is transmissible. The great majority of workers have accepted the evidence of the filterable character of the infective agency, and it is this which gives to the disease much of its peculiar interest.

The relation of the true mosaic diseases to certain other types of

[1] This paper was read at the annual meeting of the American Philosophical Society, Philadelphia, April 21, 1923.

plant disease involving chlorosis, whether with or without mottling, has not been positively determined, but, for the most part, there are some common characteristics of all to which at least passing reference must be made in the course of this paper. The relation of mosaic diseases to infectious or non-infectious "natural" variegation (yellow and green) of foliage plants also remains for detailed study. In this paper we propose to discuss more particularly some of the problems relating to infectious chlorosis with special emphasis on that type illustrated by the mosaic disease of tobacco.

The true mosaic "diseases" of dicotyledons constitute a somewhat homogeneous group, for they exhibit a blotching or mottling —defined as a mosaic—in which usually both a hypoplastic and a hyperplastic development of the tissues ensues. The mottling is very largely confined to the leaves and may be characterized by regions of lesser chlorophyll development (often a definite yellowing or chlorosis) and regions of intensified chlorophyll development. The latter has sometimes been treated as a quantitative intensification of the chlorophyll and the former as a degradation or diminution of chlorophyll. In discussing the symptomatology of such diseases it has been more the custom to emphasize as the disease the chlorotic areas; and certainly if the sugar cane, maize, and certain other monocotyledonous "mosaics" are included in the category of true mosaics, then the chlorotic areas are admittedly strongly diseased. Abnormal greenness may be, nevertheless, so characteristic that this too should have its place among the symptoms.

Among those who have studied tobacco, bean, and similar mosaics the view has been held also that the intensified green areas are primarily those of disease. In the studies of Dickson ('22) stress is properly laid, it seems to us, upon both aspects of chlorophyll change. Here, as in the earlier important work of Iwanowski ('03), it is clearly shown that in the leaves intensified greenness is correlated with, and in part (at least) due to, increased development of chlorophyllous tissues,—hyperplastic changes. This condition prevails as a part of the differentiation which is produced in young leaves, or in leaves formed after infection. There is no such result in organs already mature at

the time of infection. Woods ('00, '02), Chapman ('17), and others have also given attention to the anatomy of mottled areas. The yellow or chlorotic areas are ordinarily correlated with regions of lesser development of the chlorophyllous tissues,—with hypoplastic development. It is of some interest to note that in a typical mosaic disease of swiss chard observed by the senior writer at the Missouri Botanical Garden in 1919 the chief, if not sole, visible color effect was intensified greening in a blotched pattern. In general, there is in dicotyledonous mosaics a focal distribution of effects, and possibly it may be strictly analogous to conditions in human measles, or to the effects produced by an injection into the body of diphtheria antitoxin, or to the focal distribution of pigment in certain skin diseases. In the mosaic disease of tobacco, necrosis, as generally understood in plant pathology, does not occur.

It should not, however, be assumed that marked mottling is necessarily a symptom of these diseases, since many plants or plant species, not themselves seriously mottled, may exhibit, through infection experiments, evidence of severe attacks of the disease so far as this may be expressed through the infectiousness of their juices. Moreover, dwarfing of the general plant, spindling shoots, abscission of blossoms, and many other characteristics are typical of mosaic diseases as they are understood in certain plants, notably in the potato and in certain cucurbits.

Mosaic diseases are recognized to occur in many species of *Solanaceae* (night-shade family), *Cucurbitaceae* (gourds, squashes, etc.), *Leguminoseae* (peas, clovers, etc.), *Chenopodiaceae* (beet and spinach family), *Rosaceae* (raspberries, etc.), *Gramineae* (grasses, sugar-cane, etc.), and many others, altogether about 20 families.

There have been several possible views as to the nature of the causal agency or agencies in mosaic diseases, all or nearly all of which have been exploited, and perhaps very nearly discarded.

ENZYME THEORY

The chief adherent of the enzymic nature of mosaic disease has been Woods ('99, '00, '02). He postulated that the cause of mosaic may be found in an enzyme disturbance in which the

chlorotic condition might result from an extensive development of oxidizing enzymes. Unfortunately, this would not explain the condition prevailing where the tissues are hyperplastic, though, if found consistent, it might explain the hypoplastic relation. Moreover, the suggestion that oxidase inhibition upon diastase would explain the accumulation of starch in diseased tissues does not well apply, since the starch accumulation has been shown by Freiberg ('17) and Dickson ('22) to be in the greener areas, a finding confirmed by ourselves. Finally, Allard ('16) has convincingly demonstrated that the infective agency and oxidase are not the same, for a differential and quantitative destruction of the oxidase does not affect infectivity, whereas it is possible also to destroy the active agency in the mosaic disease and yet demonstrate oxidase action. Woods' viewpoint has been adopted also by Heintzel ('00), and in part by Chapman ('13). After criticising (Hunger, '03) the oxidase theory of Woods, Hunger ('05) regards the disease as a nutritional one possessing the peculiar property of being "physiologically autocatalytic," acting by contact and also able to regenerate itself. In some work done in this laboratory Freiberg ('17) also advocated the enzyme viewpoint rather than the more general virus effect, but he considered the enzyme to possess none of the nature of oxidases. The idea that it may be an enzyme was based in part on its absorption by talc, on the specificity of the reaction between the mosaic agency and formaldehyde, and likewise on the basis of the resistance of the body to antiseptics in general. Just how the reproduction of such an enzyme might be accomplished was not considered in detail, but was accounted for on general physiological grounds. In this connection attention was drawn to the fact that upon the injection of the toxin of *Bacillus diphtheriae* into a healthy patient, the usual pathological condition results, that is, the production of lesions characteristic of that disease, apparently with the production of additional toxin in the system.

THE BACTERIAL THEORY

In one of the earliest of the scientific reports on the mosaic disease of tobacco, that of Mayer ('86), bacteria were regarded as the causal agency, although no satisfactory proof was afforded.

Iwanowski ('03) describes the presence of bacteria-like as well as amoeba-like bodies within the tissues. The bacteria were described as intracellular, occurring in the vicinity of the cell wall, and extremely minute in size. Being the first to demonstrate the filterable character of the tobacco mosaic agency, it is rather interesting that he (Iwanowski, '92) ultimately concluded that bacteria were causally related to the disease. Strangely enough, he did not employ the skill in determining this point that he applied to other aspects of the problem. In recent years, in spite of the rapid advances in the culturing of bacteria, the bacterial view has gained few, if any, consistent adherents. A small nitrate-reducing streptococcus found in mosaic-affected tobacco is briefly referred to by Boncquet ('16, '17). Bacteria-like bodies were also identified by Dickson ('22), and he endeavored to culture the organism. By his method, bits of affected leaves were cut out, and after short disinfection intervals in alcohol and mercuric bichloride, crushed in tubes of bouillon. As clearly recognized by him there could be no certainty that surface organisms were killed. Dickson, however, secured infection by inoculation from these tubes after clouding occurred. Similar results, as he indicates, might be obtained by this method, in view of the amount of the agency originally inserted, whatever the nature of the infective particles, and the clouding with bacteria may have been entirely from secondary or surface forms.

It is rather significant that there are so few adherents of the bacterial nature of mosaic diseases. On the other hand, there is no great amount of published evidence against the bacterial viewpoint. This is certainly not wholly due to lack of effort to find bacteria, but rather to two facts: first, in at least half a dozen cases personally known to the writers, where extensive studies were made, the negative evidence was considered of too little consequence for publication; and second, acceptance of the filterable organism view tended to discourage search for bacteria. The bacterial view may be regarded at present as wholly unsustained.

THE VIRUS OR FILTERABLE VIRUS THEORY

The connotation of "virus" is fairly definite, inasmuch as the term is now generally restricted to homologize with a filterable

agency of disease. In the present paper we shall use the term virus in the general sense just referred to. The ability of the agency or "organism" to pass through the pores of a standard Berkefeld or Chamberland filter is the usual criterion. Some would undoubtedly define a virus as an ultramicroscopic organism, probably of bacterial nature. This, however, was not the final view of Beijerinck ('99a) who postulated a "contagium vivum fluidum" as the cause of the mosaic disease of tobacco. While his agar filtration studies may now be regarded as inadequate, the capacity of the infectious agencies of several mosaic diseases to pass through certain standard filters under certain conditions has now been demonstrated by Iwanowski ('92), Beijerinck ('99), Allard ('16), Doolittle ('20), Duggar and Karrer ('21), and others. After summarizing an interesting study of the properties of the virus of tobacco mosaic Allard ('16a) is convinced that "there is every reason to believe that it is an ultramicroscopic parasite of some kind." If this evidence of the "filterable" nature of the disease is admitted, it would bring the causal agency into a class possibly composed of a rather miscellaneous group of bodies, since there are well-known analogies in the agents of animal disease. The "virus" view in one form or another has been widely held, but the favorite idea has been an ultramicroscopic organism.

THE AMOEBA OR PROTOZOAN THEORY

It has been pointed out that both Iwanowski and Hunger drew attention to the presence in mosaic plants of bodies which they interpreted as amoeba-like. These, however, were of relatively infrequent occurrence. More recently, in a study of the mosaic disease of sugar cane in the tropics, Matz ('19) has found certain cells of the affected tissue filled with a granular matter. Using reliable cytological methods, Kunkel ('21) confirms the presence of such cells in a mosaic disease of corn, to which, however, he seems very justly to attach no significance. Kunkel does find in the cells of diseased corn peculiar plasma-like bodies in the vicinity of the nucleus. He has also been able to distinguish similar structures in the "diseased" areas of *Hippeastrum* (Kunkel, '22) also affected with a mosaic disease. This is a very clean-cut

demonstration of a plasma-like body, but whether or not it may be a modified cell structure, a pathological by-product, a colony of granular bodies, or something else, is not yet clear, nor is its significance in relation to the "mosaic" disease of these monocotyledons known.

A study of tobacco mosaic in Sumatra by Palm ('22) brings casual reference to some abnormal structures. He refers very briefly to cytological work on this mosaic and mentions the occurrence of corpuscular bodies more opaque than the general protoplasm. Likewise, he notes the occurrence of "a second foreign cell element, consisting of extraordinary, small granules." With this hazy evidence he proceeds to relate the bodies to the "so-called corpuscles of Gardner" and concludes that a "Strongyloplasma" species must be considered as the cause of the disease. Indeed, he designates the "organism" *Strongyloplasma Iwanowskii*, promising a more extended publication.

The sensation of the joint meeting of the Botanical Society of America and the American Phytopathological Society at Boston in December, 1922, was a report by Nelson on "The Occurrence of Protozoa in Plants Affected with Mosaic and Related Diseases." The stage was well set for such an announcement. The titles of several papers arranged for that same meeting indicated the finding of unusual structures in the cells of several plants affected with mosaic-like diseases. The careful work of Kunkel ('21, '22) referred to earlier in this paper; the observations of Matz ('19), Palm ('22), and Dickson ('22); the attention recently bestowed upon the existence in the spurge and milkweed families (Lafont, '10; França, '20; and Mesnil, '21) and other dicotyledons (Franchini, '22, '22 a-g) of flagellates normal to the latex tubes;—these considerations all served to establish an atmosphere on that stage exceedingly favorable or impressionable in respect to protozoology. Under such conditions Nelson described or presented upon the screen in the form of photomicrographs evidence for the existence in bean mosaic of 6 principal forms or types of a protozoan organism alleged to occur in the phloem of a diseased plant. It is impracticable here to take the time to indicate the characteristics of most of these types of flagellates described. The paper has since appeared as a technical bulletin of the

Michigan Agricultural Experiment Station (Nelson, '22). Besides the bean mosaic, similar diseases of clover and tomato, and the leaf roll of potato are reported replete with protozoans. It may not have appeared remarkable at the time the paper was presented but it is significant now that Nelson gave no picture of the conditions in the comparable cells of healthy plants. In two places in the printed paper (Nelson, '22) he refers to healthy tissue, one reference being to the potato, where he says, in part, "No organisms have been found in the sieve tubes of these plants in all the slides examined;" whereas in a general discussion of relationship he affirms that "the finding of definite protozoan organisms in constant association with mosaic plants and their absence from healthy ones indicate that they are probably the factor so long sought as the cause of these diseases."

We have endeavored to supplement this work with an elaborate cytological study of healthy and diseased tobacco and tomato tissue, healthy bean tissue, and healthy cucurbit tissue. In the Solanaceous plants we find in the phloem and in other elongated cells of perfectly healthy individuals precisely the same bodies that are found in diseased tobacco and tomato plants. The number of cells with such inclusions is not great. The most characteristic of these bodies are often sinuous, or screw-like, also of other types. They are usually homogeneous and often appear to be waxy in nature. Some supernumerary nuclei or cytoplasmic aggregates are also observed, and the remains of plastids may be associated with these. We have not studied diseased bean tissue, but in healthy tissue the long cylindrical, ovoidal, or elliptical bodies are generally homogeneous in character, centrally disposed, and frequently associated with cytoplasmic strands, the latter giving the appearance of one or more flagellae at the ends. It seems apparent that these particular bodies are those that have been described by Strasburger in normal sieve tissue. In certain cucurbits, notably in *Chayote edule*, disintegrating plastids in cells undergoing rapid elongation present the appearance of organisms of various types, all more or less nodose. It seems unnecessary to describe these bodies further in the present connection. It is clear that the peculiar structures portrayed by Nelson are all to be found, but our claim

is that essentially all of these may be paralleled in perfectly healthy tissue. Moreover, the relations of these bodies seem in no way to suggest flagellates that may be normal to the tissues, whether diseased or healthy. From our studies we are convinced that these "flagellates" are made up of several factors, and while we have not attempted a careful micro-chemical examination, nor made a complete study of the developing tissues, such possibilities as the following may be noted: elongated masses of gummy material long known to be characteristic of certain sieve tissues; cytoplasmic aggregations or areas of contraction possibly associated with disintegrating plastids; elongate, accessory, and perhaps disintegrating nuclei; and homogeneous aggregates of unknown origin, possibly of waxy nature.

The importance of the problem justifies the feeling that a complete reinvestigation of these cell phenomena is being pursued by many others, and that out of it may come some compensating observations that will throw light rather than shadow on the nature of the mosaic diseases.[1]

ULTRAFILTRATION EXPERIMENTS

The fact so frequently confirmed that the agency of mosaic disease passes freely through the pores of the average Berkefeld or Chamberland filter did not establish, prior to 1921, the size of the infective agency further than to indicate that it is considerably less than that of the usual plant or animal pathogen. It sufficed merely to relate the agency to filterable organisms. In order ultimately to determine more accurately the relations of this agency it seemed essential to make a detailed study of its size relations. This was done by the present writers (Duggar and Karrer, '21), and reference to this work is a necessary preliminary to the further results which will be reported and to the theoretical considerations which we wish to present.

The work referred to consisted first in securing graded series of ultrafilters, some of which should permit the infective particles to pass freely (as shown by the infectiousness of the juice) and

[1] Since the oral presentation of this paper, much additional light has been thrown upon the distribution and relations of these abnormal bodies described by Nelson, and similar structures, in a series of papers published in Phytopathology, Vol. 13, No. 7, 1923, by the following authors: (1) Kotila and Coons; (2) Dcolittle and McKinney; (3) Kofoid, Severin, and Swezy; and (4) Bailey.

others with pores or lacunae so fine as to prevent or greatly inhibit the passage of such particles. A second phase of the work involved a careful technique in the use of the ultrafilters. A third phase required the inoculation of healthy plants with the various filtrates obtained in order to determine the percentage of dilution of the particles, if possible. Finally, some method of standardization of the filters was necessary whereby their capacity to permit or prevent the passage of particles might be related to colloidal particles of known, or approximately known, sizes. It will be unnecessary to go into the details of these experiments. Two aspects of the results require emphasis. It was possible to find a filter, in this case a cylindrical, porcelain atmometer cup, which in a given interval of time, at a given pressure, and at the reaction of the diseased tobacco juice, permitted only a relatively small number of the infectious particles to pass through. Considerable dilution of the juice from the standpoint of these particles was therefore effected. This was shown by a reduction in the incidence of infection from 90–100 per cent in the usual controls to 5–20 per cent in the case of the porcelain filter only partially permeable to the infective particles.

Standardization of the filters was accomplished by the use of hydrophilic colloids of biological origin. These were selected in preference to sols of inorganic origin, such as gold sols, because of possible greater complications (when employing the latter) arising from electrical relations. The series of organic compounds employed included casein, gelatin suspensions, lactalbumin, hemoglobin, and dextrin. Fortunately, this series sufficed. The results indicated that the hemoglobin content of a standard hemoglobin solution prepared from fresh ox blood was diluted to a very considerable degree in passing through the same filter which obstructed to a large degree the passage of the infective particles. In experimenting further with substances on either side of hemoglobin, in reference to size range, it was clear that from such filtration experiments the deduction must be made that the infective particles of mosaic disease approximate in size those of a fresh 1 per cent hemoglobin solution.

The best data on the size relations of hemoglobin particles indicated a diameter of approximately 30 $\mu\mu$. It is presumable

that we are dealing in the case of a colloidal solution with particles and not with molecules. This particle size is to be compared with an average short diameter of about 1000 μμ for many pathogens.

If we are dealing with an organism, that is, an organized ultra-microscopic individual of approximately 30 μμ in diameter, its life relations must be very different from those of an organism whose volume relations are to this as 37000 to 1 or about 1,000,000 to 26. This would be the relation between the average bacterial plant pathogen and the mosaic virus. Assuming a complex organization, many theoretical questions would arise for consideration. Among these might be mentioned perhaps above all that of the surface tension conditions in such a structure, also the possibility of organization at all (membrane existence, etc.) as now comprehended.

The filtration work has been repeated with scrupulous care and it has led to results similar to those above described,—invariably pointing to an infectious particle with a size approximating that of fresh 1 per cent hemoglobin. A question which then forces itself upon the attention is: What is the peculiar nature of such a particle? To arrive at a tentative answer to this question, it would be necessary to consider all known properties of the agency, to analyze the data already carefully worked out, to plan many experiments of an entirely new type with a view to determining the behavior of the body concerned, and to contrast the inception and course of the mosaic disease or related phenomena of chlorosis in other plants. As far as possible it would be essential to examine also any possible relations of the viruses of animal diseases that may assist in one or more general interpretations.

Under the most favorable growing conditions the period of incubation of the tobacco mosaic is from 10 days to 2 weeks. By period of incubation, in this connection, is meant the time required for the development in the young leaves of the infected plant of unmistakable symptoms of mottling. In this interval of time the infective agency is widely distributed in the plant. It is not confined to the leaves (young) capable of exhibiting pronounced or favorable mosaicing, but may be found in older leaves, roots, etc. It has in reality a phenomenal power of "migration" from cell to cell,—a power none the less pronounced

even if the vascular system should be shown to constitute one of the paths of this migration. Moreover, the power of migration is not a matter which may be easily determined.

The rapid and almost complete distribution of the organism in the tissues accords well perhaps with a body minute in size and attenuate in form, or else a body so fluid in character as to be capable of assuming an extremely attenuate form. A living structure so pliable and attenuate might be expected to be sensitive to reagents and conditions. To a considerable degree this sensitiveness is not true of the virus of mosaic. In our experiments it resisted the usual procedure of dehydration by means of acetone and alcohol. Modifications of the Buchner method for the extraction of enzymes (zymase) from yeast cells were applied to a pulp of fresh, diseased, leaf tissue ground with very fine quartz sand, 5 parts of the former to 1 of the latter. Three series of experiments were arranged. In the first, the pulp was treated with full-strength acetone. There were 2 treatments, each of 3 minutes, the tissue being drained after the first addition of acetone and then fresh acetone applied for an equal interval. Finally, the material was dried as promptly as possible under an electric fan. In the second case the dehydration treatment consisted in the addition of 95 per cent alcohol for a 3-minute interval, followed by pure acetone, and finally dried, as above. In the third case the treatment was 95 per cent alcohol, followed, in the same intervals as before, with 98 per cent alcohol, and finally dried. After 3 days these residues were extracted with water, each for 1 hour, using about 10 parts of water to 1 of the dried material. After the filtration of each extract through cotton, 20 plants were inoculated with each extract, and suitable (20) controls were maintained. These plants were under favorable growth conditions, and they exhibited the symptoms of disease promptly. At the end of 19 days, 1 healthy plant only remained in each set. All uninoculated controls remained healthy, and the incidence of disease in the control which was inoculated with diseased juice was likewise 19.

When, however, the amount of alcohol and acetone was greatly increased in relation to the bulk of material used (approximately 200 times as much), the incidence of infection was low, showing

that the infective particles do not withstand complete dehydration.

A study of the effects of longer exposure to various grades of alcohol has been carried out at some length by Allard ('16a), who found that relatively speaking the infective properties are quickly destroyed by the higher strengths of ethyl alcohol. He indicates destruction by 80 per cent alcohol in 30 minutes. On the other hand, in later work, he (Allard, '18) has shown some striking resistance of the infective agency to the weaker grades of alcohol. We may note a few instances. Kept in 25 per cent alcohol 34 days and then inoculated into the host, 7 out of 10 plants developed mosaic; this, however, is obviously exceptional, since in another test the virus kept in 25 per cent alcohol 199 days yielded 7 out of 10 diseased plants; in 50 per cent alcohol after 40 days no disease was induced; in 50 per cent alcohol after 35 days 4 out of 10 plants became diseased. With another sample of the virus in 50 per cent alcohol for about 5 days only 2 plants were diseased after inoculation from this material.

From a comparison with active cells in the vegetative condition it will be seen that these results indicate a high degree of resistance since the average bacterial cell may be injured after 24 hours by a concentration of from 5 to 10 per cent alcohol. Moreover, the yeast cell, which shows a specific tolerance of alcohol concentration, is itself injuriously affected by more than about 15 per cent alcohol. If the comparison is made with the tolerance of the spores of certain species of bacteria, we shall find that the infective particles of mosaic are less resistant. Feeling that it was unwise to accept some of the data which have been published on this point, we made a study of the tolerance of the spores and vegetative cells of the hay bacillus, *Bacillus subtilis*.

In the duplication of this work the senior author was assisted by Dr. H. R. Rosen. One cubic centimeter of a dense infusion of this organism was placed in a series of alcohols diluted with a decoction of tobacco juice so as to get respectively 10, 20, 30, 40, and 50 per cent alcohol, and similar concentrations of acetone. At intervals up to 10 days, streak cultures from these concentrations of the disinfectant yielded in each case continuous growth of the organism. Similar cultures were made with a young

culture of the bacillus in which, of course, there were relatively few cells in the spore condition. In the latter case very few colonies appeared after the third day. In a repetition and amplification of this work, the tobacco extract was not employed, and the organism was suspended in concentrations of alcohol, as follows: 10, 20, 40, 60, and 100 per cent, with controls in bouillon and in distilled water. From all those cases in which the spore suspension was employed, a profuse growth was obtained on every streak culture from 10 to 99 per cent alcohol, with apparently no lessening of the intensity of growth as between 10 per cent and 99 per cent. In the case of the acetone-treated material, profuse growth was attained at 10, 30, and 60 per cent acetone with some indication of less intense growth in absolute acetone.

Following the above observations, made the third day, it was determined to make isolation cultures after the tenth day, and these were accordingly arranged and a careful count made as the colonies appeared. There was a progressive diminution in the number of cells alive from the 20 per cent to the 99 per cent alcohol. For that whole interval, however, this decrease amounted to only about nine-tenths of the organisms present in the infusion. There was scarcely any diminution as between 10 per cent acetone and 60 per cent, but in absolute acetone the number of organisms was considerably reduced. These data confirm the statement previously made to the effect that the virus of mosaic is less resistant than certain spore forms of the bacteria. This, however, is not surprising, for whatever may be the nature of the virus, many colloids lose to a considerable degree their hydrophilic character when treated with strong alcohol. The point of interest, therefore, is more particularly the nature of the bacterial spore which permits survival in the high concentrations discussed, a problem rather apart from our specific investigation.

EFFECT OF GRINDING ON THE INFECTIVITY OF THE TOBACCO VIRUS

Inasmuch as the thermal tolerance and the resistance toward dehydrating and disinfecting agents, while suggestive, did not seem to set off this virus as possessing properties peculiarly distinctive, it seemed particularly desirable, in view of the size

relations, to determine the influence of long-continued grinding under conditions which are generally effective in disrupting living cells. A final series of experiments in this field will be sufficient to indicate the relations encountered. It should be indicated, however, that this series is in accurate accord with less extensive work previously undertaken to determine the same point. The grinding was carried out in an agate mortar with motor-driven, excentrically arranged pestle, the usual device employed in grinding bacterial cultures. Equal amounts by weight of fresh leaf material and diatomaceous earth were used.

INOCULATION EXPERIMENTS WITH FINELY GROUND MATERIAL FROM DISEASED TOBACCO LEAVES. INTERVAL, 3 WEEKS

Nature of inoculum	Total diseased after 4 weeks (ten plants inoculated)
Ground 3 hours	8 plants diseased
Ground 9 hours	6 plants diseased
Control, no inoculation	None diseased
Control, fresh dis'd. juice	7 diseased

While there has been some inconsistency in the data from other grinding experiments they point in general to one conclusion, namely, that the virus is highly resistant to protracted grinding with diatomaceous earth when the virus is ground with fresh leaf pulp. It is less resistant when filtered through porous cups, then mixed with diatomaceous earth, and ground for 9 hours. The presence of leaf material acts to prevent the greater injury. In order that these experiments may be significant it is necessary to compare the grinding of the tobacco virus with that of a species of bacteria.

For this purpose also we have employed *Bacillus subtilis* in the spore condition. Two cc. of a 22-day-old culture in bouillon (rich in spores) were thoroughly mixed with 2 gms. sterile diatomaceous earth in a petri dish. This mixture was then dried, being protected during drying by sterile paper bags. It was then subjected to grinding for the same intervals as previously employed, namely 3, 6, and 9 hours. All possible care was taken to prevent contamination of the material, but some sporadic contamination was unavoidable. As the grinding progressed, a sample

was taken after each interval, the sample being removed from directly beneath the pestle. This was placed in a sterile weighing flask and weighed. One-tenth gm. of the sample was diluted to 9 cc., giving a dilution of 1:100. From this, other dilutions up to 1 to 10^6 were made, and poured plates were arranged from these dilutions. A sample from the original bouillon culture mixed in the same proportion with the diatomaceous earth, without grinding, was plated out at similar concentrations. Duplicate cultures were made in every case. The result of these experiments indicated that even after grinding the spores of *Bacillus subtilis* for only 3 hours very few remained viable, an average of 32 per culture at a dilution of 1:100, at which dilution the control showed innumerable colonies. After 6 hours of grinding the viable spores averaged 2 per culture, and no greater dilution yielded any colonies whatsoever. After 9 hours a single colony appeared at a dilution of 1:100, and no colonies at greater dilutions. Grinding was therefore thoroughly efficient in killing the spores of bacteria.

We have carried out a variety of experiments on temperature relations, the effects of disinfectants, the action of light, etc., without securing any results that indicate unusual peculiarities of the mosaic. *In vitro* studies of the mosaic agency have likewise failed thus far to give any evidence of change in the culture solutions indicative of the activity of living organisms. In addition to these lines of research we have also undertaken extensive experiments, beginning in the winter of 1921–22, in the filtration of bacteria, with the idea of determining the capacity of such organisms to pass filters when apparently the spore or cell sizes were greater than the diameters of the pores, or lacunae, of the filters employed. These experiments have yielded results of striking interest, and in time they will be published separately. As bearing on the particular problem in hand, however, no application of the investigation seems possible, both because of the inactivity of the mosaic "virus" and the lack of evidence of any stage of the latter of microscopic dimensions.

In endeavoring to arrive at something more concrete than the mere name "virus" to explain the general nature of the mosaic disease agency, we need to recall many facts bearing upon somewhat related phenomena. From the investigations of Lindstrom

('18) the inheritance of a number of chlorophyll types is shown to be strictly Mendelian. These types involve various degrees of striping and cases in which the chlorophyll is almost or entirely suppressed, with the production of white, virescent, or yellow seedlings. Passing from these normally inherited color characteristics to those which are infectious, such cases as those of the variegated *Abutilon* and the striped *Ligustrum*, worked upon by Baur ('06, '07, '08), come up for consideration. In these cases, it will be recalled, there is a characteristic pattern of color, but there is no noticeable tissue modification. Transmission is by grafting only.

The types just discussed, without graft-infection experiments to demonstrate their peculiarities, would be considered "normal" variations. The infectiousness, however, is precisely that which was found by Erwin Smith to prevail in peach yellows. "Peach yellows" is, in part, a chlorotic disease, but it gradually leads to severe injury and ultimately death of the peach tree. The disease is not transmitted by pollen nor, so far as known, by seed, but a diseased scion will convey the disease in time to a healthy stock. This disease has been considered by many to possess a highly infectious nature, but of this infectious character the senior writer has been wholly unable to find any authentic proof. Statements indicating that it may "sweep an orchard in a few years," when followed up are found to be equally as well explained by the possibility that all the stock came from a single nursery at the same time. Scions from the same tree may have been employed. This disease, moreover, is rather closely localized in a narrow climatic zone. The claims of sporadic appearance of the disease in regions far south of Michigan and Delaware have in very few, if any, cases been adequately verified, especially since the water-shoots arising in clusters from severely headed-back or winter-injured trees possess many characteristics of yellows, clearly recognized, however, as water-shoots by the expert.

One should include in the graft-transmissible forms reference to the recent work of Blakeslee ('21), in which a graft-infectious disease of *Datura* resembling a vegetative mutation is discussed and its behavior in heredity clearly set forth. This disease has

been known as the *Quercina* form. It is not artificially transferable except by grafting, but certain other species of *Solanaceae* are susceptible through grafting. It is transmitted by seed to about 79 per cent of the offspring when pollinated with normal plants.

The case of the curly-top of sugar-beets, which is generally assumed to be related to mosaic disease, is peculiar in that no infection by diseased juice can occur until the juice has passed into the body of *Eutettix tenella*, in which it must remain a definite time interval, or incubation period, before being infectious to beets.

In this case not even grafting has been successful according to the more recent reports. In still another category with respect to infectiousness may be included the case of mosaic in sugar-cane, poke-weed, and other plants in which insect transfer is the more effective method yet found.

The well-known cases in tobacco, bean, cucurbit, and other plants wherein the transfer of juice from diseased to healthy plants, whether by aphids or by needle prick, is sufficient to reproduce the disease,—these are more closely related, as to infectiousness, to ordinary bacterial or other parasitic diseases. Detailed experiments by us confirm the view that the virus of tobacco and of related mosaic diseases do not pass readily, if at all, through uninjured surfaces. We have tested this by spraying the diseased juice on the leaves, also by placing the diseased juice in glass cells sealed to leaf surfaces for 24 hours or more. Under such conditions the virus is practically inert.

It is suggestive that in the tobacco mosaic, the tomato mosaic, and many others, the gametes do not seem to possess the virus; at least the embryo arising from the fused gametes is not diseased, while the seed-coats are. It is conceivable that the reduction division is concerned in the elimination of the disease, a possibility which, if established, would be significant. The case of the bean is, however, an apparent exception, though the possibility of infection after early embryonic development is not excluded.

Time prevents a more complete discussion of the bearing of these facts, but the trend of the evidence seems to indicate that

we have here a group of viruses which, apart from the cell, are as inactive as any colloidal particle lacking that correlated organization which is characteristic of cell life. Within the cell such a virus possesses unusual activity, obviously. So far as resistance to environmental conditions is concerned, we have to admit frankly that there may be no great difference between a living cell, and enzyme, and many types of biocolloids, but, on the whole, the mosaic virus behaves as if it were a biocolloid, yet one endowed with the power of reproduction. Now it has been frequently suggested in the literature that all these discussions as to the nature of a virus are unnecessary, since we may just as well take the easier, simpler view, and call a virus an ultramicroscopic organism. The facts are just a little out of line, if viewed in their broadest aspects; and the fascination is to go on and perhaps ultimately get a satisfactory explanation, or arrive at what may be an acceptable theory.

We cannot forget that important contributions have been made almost within the year. The d'Herelle phenomenon is itself a remarkable discovery. Here is a filterable body—call it what you like—appearing in the excretions of dysentery, which, placed in contact with the bacterial culture, is lethal to the culture; and at the same time the body propagates itself.

Again, if all viruses are minute bacteria, why are there no analogues of such microorganisms as saprophytes? Why are there none in butter, in milk, in soil, in fermentation phenomena of one type or another? While some "indications" of the existence of filterable organisms in such environments have been reported, it must be admitted that all changes in such substrates have been related to organisms that are not ultramicroscopic, and no such parallel in nature has been clearly demonstrated so far as I am aware. There are, of course, many diseases induced by extremely small microorganisms but the question is: Have we not already reached the point where our technique may always make evident some stage of such organisms? Are any truly ultramicroscopic organisms culturable? With agencies of the mosaic virus type we have made no progress, possibly because progress is not attainable by culture methods and by microscopic vision.

In respect to size relations some pertinent questions also arise. Is it, for example, possible that a protoplasmic particle may be as small, in small diameter, as a hemoglobin particle, remembering that the former must carry the properties of surface and central plasma and of nucleoplasm—indeed of all the characteristics of an individual? This particular question does not seem to us to be affected by any consideration of the magnitude of the long diameter of the individual. Such an individual could not presumably penetrate cell walls, and its rapid spread through the tissues would be dependent upon bridging protoplasmic fibrils between the cells.

If one is compelled to admit the existence of an organism of the size relations above referred to, it would seem necessary with the data at hand to conceive of it as a flagellum-like creature with perhaps a temporary hook-up of molecules or colloidal particles, conceivably with no true bordering membrane and no restricted endometabolism. The supposition that the organism might be of an extremely fluid nature would perhaps be equally unsatisfying.

Taking into consideration all the facts, we cannot avoid the impression, tentatively, that the causal agency in mosaic disease may be, in any particular case, a sometime product of the host cell; not a simple product such as an enzyme, but a particle of chromatin or of some structure with a definite heredity, a gene perhaps, that has, so to speak, revolted from the shackles of coordination, and being endowed with a capacity to reproduce itself, continues to produce disturbance and "stimulation" in its path, but its path is only the living cell.

BIBLIOGRAPHY

Allard, H. A. ('16). The mosaic disease of tomatoes and petunias. Phytopath. **6**: 328–335. *f. 1–2.* 1916.

————, ('16a). Some properties of the virus of the mosaic diseases of tobacco. Jour. Agr. Res. **6**: 649–674. *pl. 91.* 1916.

————, ('16b). A specific mosaic disease in Nicotiana viscosum distinct from the mosaic disease of tobacco. *Ibid.* **7**: 481–486. *pl. 55–56.* 1916.

————, ('18). Effects of various salts, acids, germicides, etc., upon the infectivity of the virus causing the mosaic disease of tobacco. *Ibid.* **13**: 619–637. 1918.

Baur, E. ('06). Weitere Mitteilungen über die infektiose Chlorose der Malvaceen und über einige analoge Erscheinungen bei Ligustrum und Laburnum. Ber. d. deut. bot. Ges. **24**: 416–428. 1906.

————, ('07). Über infektiose Chlorosen bei Ligustrum, Laburnum, Fraxinus, Sorbus und Ptelea. *Ibid.* **25**: 410–413. 1907.

————, ('08). Über eine infektiose Chlorose von Evonymus japonicus. *Ibid.* **26**: 711–713. 1908.

Beijerinck, M. W. ('99). Bemerkung zu dem Aufsatz von Herrn Iwanosky über die Mosaikkrankheit der Tabakspflanze. Centralbl. f. Bakt. II. **5**: 310–311. 1899.

————, ('99a). Ueber ein Contagium vivum fluidum als Ursache der Fleckenkrankheit der Tabaksblätter. *Ibid.* **5**: 27–33. 1899.

Blakeslee, A. F. ('21). An apparent case of non-Mendelian inheritance in Datura due to a disease. Nat. Acad. Sci., Proc. **7**: 116–118. 1921.

Boncquet, P. A. ('16). The presence of nitrites and ammonia in diseased plants. I. Its significance with regard to crop rotation and soil depletion. Am. Chem. Soc., Jour. **38**: 2572–2576. 1916.

————, and Mary Boncquet ('17). *Ibid.* II. Oxidases and diastases; their relation to the disturbance. *Ibid.* **39**: 2088–2093. 1917.

————, ('17a). Bacillus morulans, n. sp. A bacterial organism found associated with curly top of the sugar beet. Phytopath. **7**: 268–289. *f. 1–7.* 1917.

Chapman, G. H. ('13). "Mosaic" and allied diseases, with especial reference to tobacco and tomatoes. Mass. Agr. Exp. Sta., Rept. **25**[2]: 41–51. 1913.

————, ('17). Mosaic disease of tobacco. Mass. Agr. Exp. Sta., Bull. 175: 73–117. *pl. 1–5.* 1917.

Dickson, B. T. ('22). Studies concerning mosaic diseases. MacDonald Coll., Tech. Bull. 2: 1–125. *pl. 1–8.* 1917.

Doolittle, S. P. ('20). The mosaic disease of cucurbits. U. S. Dept. Agr. Bull. 879: 1–69. *pl. 1–10.* 1920.

Duggar, B. M., and Joanne L. Karrer ('21). The sizes of the infective particles in the mosaic disease of tobacco. Ann. Mo. Bot. Gard. **8**: 343–356. 1921.

França, C. ('20). La flagellose des Euphorbes. Inst. Pasteur, Ann. **34**: 432–465. *pl. 13–14. f. 1–2.* 1920.

Franchini, G. ('22). Amibes et autres protozoaires de plantes à latex du Muséum de Paris. (Note préliminaire). Bull. Soc. Path. Exot. **15**: 197–203. 1922. (Rev. in Bot. Abstr. **12**: 439–440. 1923.)

————, ('22a). Flagellose du chou et des punaises du chou. *Ibid.* 163–165. *f. 1.* 1922. (Rev. in Bot. Abstr. **12**: 440. 1923.)

————, ('22b). Nouvelles recherches sur les trypanosomes des Euphorbes et sur leur culture. *Ibid.* 299–303. *1 f.* 1922. (Rev. in Bot. Abstr. **12**: 440. 1923.)

————, ('22c). Remarques à propos de la note de M. França sur la flagellose des Euphorbes. *Ibid.* 205–207. 1922. (Rev. in Bot. Abstr. **12**: 441. 1923.)

————, ('22d). Sur une amibe des figuiers de plein air de la region parisienne et sa culture. *Ibid.* 287–292. *f. 1–3.* 1922. (Rev. in Bot. Abstr. **12**: 441. 1923).

Franchini, G. ('22e). Sur un flagelle de Lygaeide (Crithidia oxycareni n. sp.). *Ibid.* 113–116. *f. 1.* 1922. (Rev. in Bot. Abstr. **12**: 441. 1923.)

————, ('22f). Sur un flagellé nouveau du latex de deux Apocynées. *Ibid.* 109–113. *f. 1.* 1922. (Rev. in Bot. Abstr. **12**: 442. 1923.)

————, ('22g). Sur un trypanosome du latex de deux espèces d'Euphorbe. *Ibid.* 18–23. 1922. (Rev. in Bot. Abstr. **12**: 442. 1923.)

Freiberg, G. W. ('17). Studies in the mosaic diseases of plants. Ann. Mo. Bot. Gard. **4**: 175–232. *pl. 14–17.* 1917.

Heintzel, K. ('00). Contagiöse Pflanzenkrankheiten ohne Microben unter besonderer Berücksichtigung der Mosaikkrankheit des Tabaksblatter. Inaug. Diss. Erlangen. 1900. [Original reference not seen.]

Hunger, F. W. T. ('03). Bemerkung zur Woods'schen Theorie über die Mosaikkrankheit des Tabaks. Inst. Bot. Buitenzorg, Bull. 17: 1–9. 1903.

————, ('05). Neue Theorie zur Ätiologie der Mosaikkrankheit des Tabaks. Ber. d. deut. bot. Ges. **23**: 415–418. 1905.

Iwanoski, D. ('92). Über zwei Krankheiten der Tabakspflanzen. Land. u. Forstwirtsch. **1892**. (Rev. in Beih. Bot. Centralbl. **3**: 266–268. 1893.)

————, ('03). Über die Mosaikkrankheit der Tabakspflanze. Zeitschr. f Pflanzenkr. **13**: 2–41. *pl. 1–3.* 1903.

Kunkel, L. O. ('21). A possible causative agent for the mosaic disease of corn. Hawaiian Sugar Planters' Assoc., Bull. Exp. Sta. **3**: 1–15. *pl. 4–15. f. 1–3.* 1921.

————, ('22). Ameboid bodies associated with Hippeastrum mosaic. Science N. S. **55**: 73. 1922.

Lafont, A. ('10). Sur la presence d'un Leptomonas parasite de la classe des Flagellés dans le latex de trois Euphorbiacées. Inst. Pasteur, Ann. **24**: 205–209. 1910.

Lindstrom, E. W. ('18). Chlorophyll inheritance in maize. Cornell Univ. Agr. Exp. Sta., Mem. 13: 7–68. *pl. 1–5.* 1918.

Matz, J. ('19). Infection and nature of the yellow stripe disease of sugar cane. Dept. Agr. Porto Rico, Jour. **3**: 65–82. 1919.

Mayer, A ('86). Ueber die Mosaikkrankheit des Tabaks. Landw. Versuchs-Sta. **32**: 451–467. *pl. 1.* 1886. (Rev. by E. F. Smith in Jour. Myc. **7**: 382–385. 1894.)

Mesnil, F. ('21). La "flagellose" ou "Leptomoniase" des Euphorbes et des Asclepiadiacees. Ann. Sci. Nat. Bot. X. **3**: XLII–LVII. *f. 1–4.* 1921.

Nelson, R. ('22). The occurrence of protozoa in plants affected with mosaic and related diseases. Mich. Agr. Exp. Sta., Tech. Bull. 58: 1–30. *f. 1–18.* 1922.

Palm, B. T. ('22). De Mosaiekziekte van de Tabak een Chlamydozoonose. Bull. von het Deliproefstation te Medan-Sumatra 15: 1–10. 1922.

Woods, A. F. ('99). The destruction of chlorophyll by oxidising enzymes. Centralbl. f. Bakt. II. **5**: 745–754. 1899.

————, ('00). Inhibiting action of oxidase upon diastase. Science N. S. **11**: 17–19. 1900.

————, ('02). Observations on the mosaic disease of tobacco. U. S. Dept. Agr., Bur. Pl. Ind. Bull. 18: 1–24. 1900.

CITRIC ACID AS A SOURCE OF CARBON FOR CERTAIN CITRUS FRUIT-DESTROYING FUNGI[1]

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INTRODUCTION

The work reported here was undertaken with the idea that the fungi which rot citrus fruits probably show some peculiar metabolic adaptations to life in such an acid environment as that furnished by the citrus fruits in general and particularly by lemons. In the progress of the work, however, the available methods, especially those for the quantitative determination of citric acid, were found to be so unsatisfactory that it was deemed advisable to spend considerable time in studying possible methods and their application to the routine work of physiological experimentation. In the first part of this paper, therefore, considerable space is devoted to the methods utilized in this research as well as to some notes on the chemistry and occurrence of citric acid. In the execution of the physiological side of the work the utilization of citric acid as a source of carbon for fungi is the special phase considered, and only passing attention is given to that other important phase, the production of acid.

It is not the intention to propose the idea that citric acid tolerance or utilization is the primary factor in the invasion of citrus fruits by fungi; in fact, due to the structure of these fruits, it is probable that a number of parasitic fungi which cannot grow in a synthetic medium as acid as the expressed juice of lemons or grapefruit are still able to rot these fruits with comparative ease. From a microscopic examination of numerous rotted fruits and from the laboratory experimentation, the idea has been gleaned that in the primary infection and rotting of citrus fruits the ability to hydrolyze cellulose, and not tolerance of citric acid, is likely

[1] An investigation carried out at the Missouri Botanical Garden in the Graduate Laboratory of the Henry Shaw School of Botany of Washington University and submitted as a thesis in partial fulfillment of the requirements for the degree of doctor of philosophy in the Henry Shaw School of Botany of Washington University.

to be one of the deciding factors. Nevertheless, for those fungi which cause the ultimate destructive decay which releases the acids contained in the juice sacs of the fruit, there must be metabolic adjustment to life in an extremely acid medium.

Discussion of Citric Acid

Citric acid (oxy-tricarballylic acid) is a tricarboxylic acid with one substituted hydroxyl group, of the structure:

$$\begin{array}{l} CH_2.COOH \\ | \\ C(OH).COOH. \quad 1\ H_2O \\ | \\ CH_2.COOH \end{array}$$

It crystallizes from concentrated solutions with one molecule of water of crystallization; but if heated too much in concentrating it turns yellow and does not crystallize, probably due to the formation of other compounds. It is extremely soluble in water but less so in most of the organic solvents, such as ether, alcohol, and chloroform. It dissociates in 3 stages, and Blasdale ('18) gives a dissociation constant of 8×10^{-4} for the first stage. Davis, Oakes, and Salisbury ('23) point out, however, that in titrating citric acid with alkali a curve corresponding to that for HCl is obtained instead of one corresponding to the titration curve for H_3PO_4; this would indicate that the di-basic and mono-basic salts are not sharply separated from each other in formation, as is the case in the similar salts of phosphoric acid. Citric acid does not possess an asymmetric carbon atom and consequently does not rotate the plane of polarized light. It forms 3 classes of salts and some mixed salts, but only the tri-basic salts are usually prepared.

Potassium citrate, used considerably in the work here reported, is a soluble, strongly alkaline salt, crystallizable with difficulty from water, owing to its high solubility, but more easily crystallized by shaking out with alcohol. Sodium citrate is even more soluble in water, but is easily crystallized out as fine crystals by shaking an aqueous solution of the salt with 95 per cent alcohol. The crystals form at the junction of the 2 liquids and slowly settle

out. Handbooks ordinarily give the sodium salt as crystallizing with 11 molecules of water but when it is crystallized out from alcohol as above described the percentage of water seems to be much lower. None of the salts of this acid with the heavy metals are quantitatively insoluble in water but most of them are less soluble in alcohol. The salts of Ca, Pb, and Ba are commonly used in analysis and will be discussed under the head of quantitative methods.

Citric acid is quite readily oxidized, its salts and the acid itself being oxidized in air at less than 200° C. The acid is decomposed by concentrated sulphuric acid, sulphuric and chromic acid mixtures, by $KMnO_4$ in acid solution, and by $K_2Cr_2O_7$ under the same conditions as for $KMnO_4$, but more slowly. The general products from such oxidations are CO_2, CO, acetone, acetaldehyde, acetic acid, and formic acid, depending on the oxidizing agent and the conditions of the reaction. On account of its high carbon content and the ease with which it is oxidized citric acid should be a fairly good source of carbon for those organisms capable of utilizing it.

QUALITATIVE DETECTION

The common methods of detection are based largely upon the fact that certain salts are less soluble in hot water than in cold, i. e., precipitates are formed on heating the solution and these disappear when the solution is cooled. Calcium citrate is commonly used in this test and the acid lead salt has been recommended by the Association of Official Agricultural Chemists ('07). This sort of method is likely to be misleading, however, in the presence of other salts or in the case of too great or too small a concentration of citric acid. Stahre's pentabromacetone method, depending upon the formation of a complicated compound, pentabromacetone, when citric acid is oxidized to acetonedicarbonic acid by $KMnO_4$ in the presence of Br, has been used extensively. This test is probably less sensitive, and much less satisfactory than that of Denigés ('98), which is based upon the formation of a complicated mercury compound with acetone. As this method was used in this work it will be given in detail and follows closely the instructions by Yoder ('11). Denigés' solution is prepared by dissolving 5 gr. of mercuric oxide in 20

cc. of conc. H_2SO_4 and diluting with 100 cc. of distilled water. To about 5 cc. of the solution to be tested, containing a small amount of citric acid, is added about 1 cc. of the mercury solution, the solution heated almost to boiling, and 2 per cent $KMnO_4$ added drop by drop with shaking. After a few drops have been added a white cloudy precipitate is formed if citric acid is present. If the $KMnO_4$ continues to be used up but no precipitate is formed it is likely that sugar, oxalic acid, or some other compound oxidized by permanganate solution more easily than citric acid, is present. If these compounds are present in small quantity, citric acid may be detected by continuing to add $KMnO_4$ solution slowly until they have been completely oxidized, when the $KMnO_4$ will react with the citric acid. If the interfering substances are present in considerable amount it may be necessary to precipitate the citric acid with barium acetate and 50 per cent alcohol, and after washing the precipitate, dissolve it in sulphuric acid, filter off the $BaSO_4$, and apply the test.

According to Yoder ('11), succinic, tartaric, and malic acids do not give this test, but aconitic acid does. Amberg and McClure ('17) stated that pyruvic, ita- and citraconic acids gave positive tests, but a large number of others tried, including tricarballylic, succinic, etc., did not give positive tests. Oxalic acid gave a white precipitate on addition of the reagent, and this must be filtered off before proceeding with the test.

QUANTITATIVE DETERMINATION

There is as yet no satisfactory gravimetric method for the determination of citric acid, though a number of such methods have been offered. The lead salt which is commonly used for the primary separation is very soluble in water, and Yoder ('11) points out that at least 3.6 volumes of alcohol are necessary for a quantitative precipitation of citric and malic acids, whereas 1 volume has usually been used.

For gross work citric acid may be precipitated from a neutral solution with calcium acetate or chloride, filtered hot, washed sparingly with hot water, and the precipitate weighed as calcium citrate or converted to $CaSO_4$ and weighed. This general method is valuable only for concentrated solutions, but is commonly

recommended. For the analysis of citric acid by precipitation as calcium citrate, L. and J. Gadais ('09) collected the filtrate and washings from the first precipitation, concentrated to a small volume, and reprecipitated, adding the second residue to the first. This is probably the best modification of the use of the calcium precipitation and increases the scope of the general method considerably.

Spindler ('03) pointed out that the calcium precipitation was not quantitative but dependent upon the volume of the solution, also that tri-calcium citrate, which is supposed to crystallize with 4 H_2O, loses water when dried at 100° C. Yoder ('11) gave the limits of concentration of citric acid for producing a precipitate with calcium acetate, without stirring or scratching the sides of the beaker, as more than 0.32 gm. of acid in 100 cc. of solution in the cold, and less than 0.32 gm. in 100 cc. of boiling solution. The writer obtained by this method an appreciable crystalline precipitate from 0.025 gm. of citric acid in 100 cc. of solution by autoclaving at 15 pounds for 20 minutes. The precipitates obtained at higher concentrations of citric acid were well crystallized by this method and easily filtered. By precipitating always from the same volume of solution it might be possible to make use of the precipitation in the autoclave satisfactorily. Calcium citrate is quantitatively insoluble in 50 per cent alcohol, but apparently no work has been done on the water content of the salt when precipitated by this method.

Creuse ('72) noted that the barium salt of citric acid was almost totally insoluble in alcohol of 0.908 sp. gr., and he offered a tentative formula for the precipitated compound of the general structure: $BaO_3.C_{12}H_8O_{11}.2\ H_2O$. He precipitated from a neutral solution in 63 per cent alcohol. The precipitate by this method is gelatinous but fairly heavy, and after standing over night can be filtered, washing mostly by decantation. Jörgensen ('07) offered a method for separating the barium salt of citric acid from that of malic acid, based upon the comparative insolubility of the citrate in 26 per cent alcohol and the greater solubility of the malate in that solvent. The separation is not sharp and requires considerable manipulation, depending on the relative concentrations of the 2 acids. Moreover, the precipitate is very

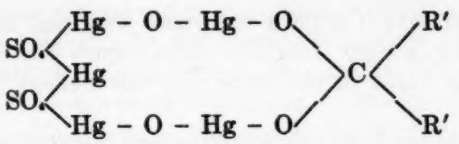
gelatinous and difficult to filter as compared with that from 63 per cent alcohol.

Several attempts have been made to base a quantitative method on the fact that $KMnO_4$ oxidizes citric acid to acetonedicarbonic acid which in turn decomposes at temperatures above 80° C. to form acetone. Jörgensen ('07) oxidized citric acid with $KMnO_4$ under various conditions but his yields of acetone were uniformly low. Fleischer ('72) obtained acetone only in the presence of mineral acid, but Jörgensen obtained it in the presence of NaOH.

Kunz ('14) modified Stahre's qualitative test and weighed the precipitate of pentabromacetone. This method has been used considerably by biochemists for determining small quantities of citric acid and has been tentatively adopted by the Association of Official Agricultural Chemists ('09). Following the lead of others Salant and Wise ('16) added Denigés' reagent to the solution to be tested, oxidized with $KMnO_4$, and weighed the mercury complex. McClure and Sauer ('22) studied both of the above methods and decided that on the whole the pentabromacetone method of Kunz was best. Both methods require tedious manipulation, are easily interfered with by substances other than citric acid, and neither gives a very good yield of the compound to be weighed.

Pratt ('12) distilled off the acetone as fast as it was formed by the interaction of citric acid and $KMnO_4$, the latter being added slowly through a separatory funnel as the reaction proceeded. The receiver flask contained Denigés' reagent. When the reaction was complete the distillate was refluxed and the precipitate weighed. Willaman ('16) modified the distillation arrangement and substituted a titration for the gravimetric determination of the acetone in the complex precipitate. This was a material improvement, since gravimetric methods are to be avoided wherever possible in the routine of physiological work where many analyses must be made.

Any method based upon the determination of the mercury-acetone complex of Denigés is necessarily empirical and is more difficult still in that the mercury-acetone complex is probably of varying as well as uncertain composition. Denigés ('98) gave for this complex the formula:



In a later publication ('98a) he stated that this formula was correct if the compound was dried at 110° C., but if dried at a lower temperature it probably had a composition represented by $[(SO_4Hg)_2.3HgO]_2.4CO.R_2$. Oppenheimer ('99) arrived at a different formula and factor for conversion to acetone and stated that there was no difference in the compound whether it was dried at 110° C. or at a lower temperature. The uncertain composition of this compound prevented a critical study of this method, since it was impossible to determine accurately the actual yield of acetone from citric acid. Theoretically 1 molecule of citric acid should yield 1 molecule of acetone, but since the rate of oxidation in some degree controlled the yield it was evident that the yield did not, in all probability, reach 100 per cent in any case. On account of this fact it was the feeling of the writer that some better method for determining the acetone might lead to a much improved method.

Pratt ('12) stated that the Messenger titration was not adapted to this method; however, Shaffer ('08) offered a method for the determination of β-oxybutyric acid based upon the oxidation of that compound to acetone by chromic acid in the presence of H_2SO_4, and the determination of acetone by the Messenger method. The interference due to the formation of volatile products from the oxidation of sugar, which were probably of an aldehyde nature, was overcome by redistillation of the first distillate, after rendering it alkaline with NaOH and adding 30 cc. of 3 per cent H_2O_2. It was at first thought that the yield was 100 per cent, but in a later paper Shaffer and Marriott ('13) showed that the yield was about 90 per cent of the theoretical and that some of the products of the oxidation of sugar were not eliminated by the redistillation, although the error from this source was negligible. Marriott ('13), studying the distillation of acetone and its determination by the Messenger method, showed that by boiling 10 minutes the acetone was completely removed to the

distillate and that if a good condenser was used the receiving flask need not be iced. Likewise, the accuracy of the Messenger titration was tested in this work and the method found to be efficient.

With this in mind the writer attempted a study of the oxidation of citric acid by permanganate, using the Messenger titration for the determination of the acetone. As only a short, straight-tube condenser was used the receivers were sometimes iced. Potassium permanganate of the concentration suggested by Pratt ('12) was used for the oxidation.

In the first experiments, using a known solution of citric acid and the conditions given by Pratt, the yields were extremely low, and although different concentrations of H_3PO_4 were used the yield never rose above 85 per cent. $K_2Cr_2O_7$ and H_2SO_4 were tried under the conditions of the Shaffer method but the yield was low. On trying H_2SO_4 with the $KMnO_4$ the yield increased to about 90 per cent and was much more constant, nor did any interference occur due to substances formed from the sulphuric acid. In using H_2SO_4 a brown precipitate was formed when the oxidation was complete and $KMnO_4$ was still added. Some of the distillations were stopped as soon as the brown color appeared, new receivers were substituted, and the distillation continued. By this method it was found that practically all the acetone yielded by the method had been collected in the first distillate, and no more than traces could be detected in the second receiver. Moreover, tests for citric acid failed when tried on the residue in the distilling flask.

As the work was being carried out with special regard to its use in culture solutions, the effect of various constituents of culture solutions was tried. The non-volatile compounds, such as sulphates and phosphates, gave no interference. Nitrates gave a substance utilizing large quantities of thiosulphate, but this compound was readily eliminated in the redistillation from alkali. Various sugars and organic acids were known to form oxidation products, and the yield of aldehyde from malic acid had been studied by Jörgensen ('07). These were studied for interference. One-half gram of malic acid was placed in each of 2 Kjeldahl flasks, and 100 cc. of water and 5 cc. of 5 *N* H_2SO_4 added. The

oxidation was carried out in the usual manner. One distillate was titrated directly by the Messenger method. On adding the alkali a white precipitate formed, the titration being only 4.0 cc. of thiosulphate on a blank of 23.15 cc. The second distillate was redistilled with 10 cc. of 10 per cent NaOH and 30 cc. of 3 per cent H_2O_2 and the distillate from this titrated. The titration was 23.15 with a blank of 23.25 cc. The procedure was repeated with small amounts of dextrose with the following results:

	Thiosulphate (cc.)
Single distillation	22.35
Redistilled	23.25
Redistilled	23.2
Blank	23.25

Shaffer and Marriott ('13) stated that a small amount of interfering substance, which was not eliminated by the redistillation, was formed when dextrose was oxidized by $K_2Cr_2O_7$ in the presence of H_2SO_4. Such a substance is not in evidence here, though it might be formed under certain conditions.

Using the apparatus and general procedure described under "Methods" a number of determinations were made on known solutions of citric acid, varying the time of oxidation, amount of mineral acid, volume of solution, and the amount of citric acid. Some of these results are given in table 1. The results varied more than if an efficient condenser had been used. Part of the results shown were obtained from a single distillation, part were redistilled.

It may be seen that the yield with H_3PO_4 as the mineral acid was around 80 per cent and very variable. Where H_2SO_4 was used the yield varied from 87 per cent to 95 per cent, depending on the volume of the solution, the amount of mineral acid, and the period of oxidation. No one of these factors, however, is as important as maintaining the same conditions for all determinations. The amount of solution, especially, seemed to affect the yield very slightly, even when raised to 200 cc., but this made distilling more difficult. On the whole, about the best conditions indicated by the results of a large number of experiments were (1) a volume of 50 to 125 cc., (2) about 1 per cent H_2SO_4, (3)

an oxidation period of 15 minutes for 100 mgms. of citric acid, and (4) a concentration of citric acid between 75 and 125 mgms.

TABLE I

QUANTITATIVE DETERMINATIONS OF CITRIC ACID UNDER VARYING CONDITIONS

Acid added, (mgms.)	Volume, (cc.)	Interval of oxidation (minutes)	Mineral acid, (cc.)	Acid recovered (mgms.)	Yield (per cent)	Remarks
			H_3PO_4*			
100	50	28	5	82.72	82.7	
100	75	21	5	84.83	84.8	
110.77	100		3	88.2	79.6	
			H_2SO_4‡			
110.77	50		1	102.9	93.0	
110.77	50		10	97.2	88.0	
110.77	50		2	101.5	91.5	
110.77	100		2	101.6	91.6	
100	75	13	5	91.87	91.8	
100	50	11	5	93.63	93.6	
100	50	14	5	93.98	94.0	
125	50	15	5	118.45	94.9	
125	50	15	5	118.9	94.96	
75	75	8	5	65.82	87.0	
75	75	12	5	67.58	90.0	
75	50	10	5	65.64	87.0	
75	50	10	5	65.64	87.0	
75	50	12	5	67.23	89.6	
75	50	14	5	67.58	90.0	
83.07			2	74.1	89.2	
55.38			2	49.67	89.5	
110.77	100		2	102.9	92.7	Oxalate added
110.77	100		2	101.9	92.1	KNO_3, redis.
110.77	50		2	105.8	95.5	.5 gm. dextrose

* 85 per cent H_3PO_4
‡ 5 *N* H_2SO_4

Proposed Method

APPARATUS

Either the short-necked 500-cc. distilling flasks described by Willaman ('16) or the ordinary 500-cc. Kjeldahl flask is suitable for the distillation and for the receiver for the initial distillation. A spiral condenser is not necessary but is desirable. The flask

is connected to the condenser with a 2-hole rubber stopper, the second hole being used for a short drawn-out dropping tube connected with a supply bottle of permanganate as described by Willaman ('16). These tubes should be drawn down equally and the tips should be small enough to release a small drop. The distilling flask should set vertically so that the liquid from the dropping tube will not strike the neck of the flask, but drop directly into the solution.

The solutions necessary are: $KMnO_4$, 0.5 gm. per liter; H_2SO_4, conc. or preferably 5 *N* (by graduate); NaOH, saturated (60 per cent); I_2, 0.1 *N* solution; $Na_2S_2O_3$, standardized 0.1 *N* solution; soluble starch, 1 per cent solution for use as indicator.

PROCEDURE

If only small amounts of interfering substances, such as sugars and organic acids, are present, enough of the solution to be analyzed to give about 100 mgms. of citric acid should be transferred (accurate pipette) into a 500-cc. Kjeldahl flask, and sufficient water to make a volume of 125 cc., and 0.75 cc. of conc. H_2SO_4 or 4 cc. of 5 *N* acid added. This should be connected with the condenser and 25–50 cc. distilled off to remove any preformed acetone or alcohol. After this preliminary distillation the receiver should be put in place and the permanganate solution dropped at about 100 drops per minute, or at such a rate as experience shows will complete the oxidation in about 15 minutes, or longer if there are interfering substances present, such as sugars. In the Kjeldahl flask arranged as the receiver 100 cc. of cold distilled water should be used, care being taken that the receiver tube is sealed off with the water.

Distillation should continue until the brown precipitate begins to form freely. The permanganate should then be stopped and distillation continued for about 3 minutes to clear out all the acetone. To the receiving flask 5 cc. of 60 per cent NaOH and 30 cc. of 3 per cent H_2O_2 is added and distillation carried out immediately, the distillate being received in cold distilled water in a flask suitable for titrating. In distilling bring to a boil slowly and then boil vigorously 10 to 15 minutes. Fifty cc. of 0.1 *N* I_2 solution and 10 cc. of 60 per cent NaOH should then be added to the receiving

flask, this being stoppered and shaken and permitted to stand for 10 minutes. Five cc. of conc. H_2SO_4 should then be added and titrated with 0.1 *N* thiosulphate, using starch solution as an indicator. The difference between this titration and the titration of a blank on the chemicals represents citric acid (1 cc. of 0.1 *N* I_2 theoretically represents 3.5 mgms. of citric acid). To the amount of citric acid found by multiplying the number of cc. by 3.5 a correction of 6 per cent should be added if the amount is over 115 mgms., 8 per cent if from 90 to 100 mgms., and 10 per cent if less than 90 mgms. It is desirable to run known amounts of citric acid and calculate the corrections for the particular apparatus and procedure used.

Where too large amounts of interfering substances are present the citric acid must be separated by precipitation. It is not necessary, however, that this separation be complete, but only that the citric acid be precipitated quantitatively and the bulk of the interfering substances removed. Two methods are suggested as convenient and practical, the one to be used depending upon the conditions under which the work is carried out.

Method 1. Precipitation as barium citrate.—To a volume of solution equivalent to approximately 100 mgms. of citric acid a drop of phenolphthalein is added and the solution neutralized with NaOH. Just enough dilute CH_3COOH (1 to 2 per cent) is supplied to destroy the pink color and sufficient barium acetate solution to completely precipitate the citric acid, then 2 volumes of 95 per cent alcohol, and the mixture shaken. If a centrifuge provided with large tubes is available the precipitation may be carried out in one of these. The supernatant liquid should be centrifuged and decanted (the precipitate comes down rapidly when centrifuged) and the residue stirred up at the bottom of the tube with a stream from a wash bottle filled with 26 per cent alcohol and centrifuged again; repeat if necessary. The precipitate should be warmed with 5 cc. of 5 *N* H_2SO_4 and a few cc. of water and the whole washed into the Kjeldahl flask and made up to 125 cc. The flask may be boiled vigorously before connecting with the condenser to remove the alcohol, this taking the place of the preliminary distillation. The precipitated $BaSO_4$ does not interfere with the distillation and oxidation.

If it is not desirable to use the centrifuge, the alcoholic solution may be warmed over the water bath, the flask or beaker being kept covered. After a few minutes of warming the precipitate will begin to flocculate out. When the solution has almost reached the boiling point of the alcohol mixture it should be set aside over night and filtered the next day. In filtering, the precipitate should be washed as much as possible by decantation, using 26 per cent alcohol, the washing completed on the filter (2 or 3 washings are sufficient), and the filter drained to remove most of the alcohol. The filter with precipitate should be transferred to a beaker, warmed with 50 cc. of water and 3 cc. of 5 *N* H_2SO_4, and washed with more warm water. Continue as previously described with the preliminary distillation or boiling.

Method 2. Precipitation as calcium citrate.—The method to be followed is essentially that of L. and J. Gadais ('09), and where a large water bath or, better still, a sand bath is available the method is very convenient. A small beaker is used instead of the crucible, and the color after neutralization with NaOH is destroyed with acetic acid and the precipitation carried out with calcium acetate instead of the chloride, giving a more easily filtered precipitate. The calcium precipitate is dissolved in 50 cc. warm water and 5 cc. of 5 *N* H_2SO_4 and transferred to the distilling flask and the filter-paper washed with 50–75 cc. of warm H_2O.

The calcium precipitation is not quite as complete as the barium precipitation, but in some ways is much more satisfactory, the precipitate of calcium citrate being crystalline and readily filtered and washed. The disadvantage lies in the slowness of concentrating the solution, after washing, to the requisite small volume.

Where only citric acid, and no other carbonaceous substances precipitated by calcium, is present, or where the only other substances so precipitated are inorganic anions, it is both more convenient and more efficient to determine the total carbon in the precipitate by the use of the Friedeman carbon apparatus as described below. This method is also very satisfactory, in many cases, with the barium precipitation, but it must be borne in mind that other organic acids are more likely to have relatively insoluble Ba salts than Ca salts, and the precipitate must be

carefully washed with 26 per cent alcohol to free it from them and the occluded sugar.

Unless oxalic acid is present in large amounts it does no harm, but where the amount is so great as to utilize large quantities of permanganate the calcium precipitation should be used and carried out first in an excess of acetic acid. The precipitate of calcium oxalate should be filtered off, the washings and filtrate neutralized, and the regular procedure followed.

It is quite possible that the autoclave may be satisfactorily made use of in connection with the calcium precipitation. A number of experiments were tried with precipitations in varying amounts of solution, 25, 50, and 100 cc., and it was found that the amount of citric acid remaining in the solution was approximately proportional to the volume. Whether this would vary in the presence of additional substances has not been determined.

OCCURRENCE OF CITRIC ACID

Citric acid is commonly known as the constituent acid of citrus fruits. Numerous writers have given figures for the acid content of these fruits, but the figures usually represent titrations of total acid, calculated as citric acid, as is the case in most of the work done on the acidity of fruit juices. However, in the case of citrus fruits the percentage of acid is so high and the presence of citric acid so well known that these figures are fairly accurate. It is probable, however, that other acids occur in small quantities in fruits of the citrus type.

Colby ('92) gave figures for the analyses of California oranges and lemons covering the crops for 2 or 3 years. The figures in table II, taken from his work, give the percentage of total sugar and acid, calculated as citric, in the juice of certain varieties.

Chace, Wilson and Church ('21) stated that California lemons contained 3 to 4 per cent of citric acid in the whole fruit (including rind). Gray and Ryan ('21), in some work on the effects of various sprays on oranges, gave some figures indicating from 0.7 to 1.5 per cent of citric acid in normal, unsprayed oranges. Collison ('13) gave figures on Florida oranges and grapefruit of various varieties. According to these, the good marketable oranges varied from 0.35 to a little more than 1.0 per cent of citric acid

and sour stock fruit had a considerably higher acidity (the acid being calculated as the anhydrous acid and not that with the usual 1 molecule of water of crystallization). Grapefruit ran from 0.8 to 1.61 per cent acidity. The total sugar figures averaged slightly lower than those cited by Colby ('92) for California fruit. All the above figures were calculated from simple titrations.

TABLE II

ACID AND SUGAR CONTENT OF CALIFORNIA ORANGES AND LEMONS

Variety	Average for	Per cent citric acid	Per cent total sugar
Navel oranges	3 yrs.	.96	10.66
Seedling oranges	2 yrs.	1.29	12.04
Mediterranean Sweet (orange)	3 yrs.	1.28	9.30
Lemons	1 yr.	6.72–8.4	1.56–2.70

The hydrogen-ion concentration of the juice of citrus fruits, extracted from thoroughly macerated pulp, was given by Haas ('17) as P_H 2.2 for lemons, 3.0 for grapefruit, and 3.8–3.9 for oranges. Bartholomew ('23) stated that the P_H of lemons varied from 2.2 to 4.4 during the course of growth, and that the average for a large number of determinations on mature lemons was 2.31.

In discussing either the total or actual acidity of citrus fruits the structure of the fruit must be taken into account. The pulp of these fruits consists of numerous small juice sacs, each of which has a definite covering or skin consisting, according to Reed ('14), of 10–12 layers of small living cells, and inside of this covering is a pulp of broken-down cells containing acid and sugar. These sacs may be easily separated from each other under a dissecting microscope without breakage and the consequent loss of acid. Reed ('14) pointed out that while the acidity of the juice contained in the juice sacs was extremely high, indeed high enough to destroy oxidases, yet the living cells of the wall of these juice sacs contain large amounts of oxidase in an active condition. From this he drew the conclusion that the acid is retained within the juice sac by a semi-permeable membrane. The living tissues are not required, therefore, to sustain any such high acidity as is found in the extracted juice.

In examining numerous specimens of partly rotted lemons and oranges attacked by various fungi it was found that the fungus had partially digested the rag, or white pithy layer inside the rind, that it had attacked the walls of the carpels, the placentae, and in some cases the outer layers of the rind, without releasing the acid from the juice sacs themselves. Examination of some oranges in an advanced state of decay showed that the walls of the juice sacs had been attacked and the juice sacs broken down, and the same was true in less degree of lemons rotted by certain fungi. This latter condition is only the result of advanced decay, however. In many cases the examination showed the rind completely digested, but the adjacent juice sacs still intact. Even where the pulp sacs were seemingly attacked, it was only when the decay had reached a very advanced stage that the fungus could be demonstrated microscopically inside the juice sacs. Oranges should present little difficulty, as far as acidity is concerned, for decay fungi, and it is probably the case that the pulp is readily destroyed, but it is improbable that the pulp of lemons can be attacked destructively until the fungus is well established in the less acid portions.

Citric acid occurs in a large number of fruits besides those included in the citrus group, and in fact citric and malic acids make up the bulk of the acids of ripe fruits. Bigelow and Dunbar ('17) summarized the work on the acidity of fruits and added data of their own. Besides citrus fruits, most berries were found to contain citric acid, and this was especially true of cranberries, which are extremely acid. Tomatoes, cantaloupes, one variety of pear, and a number of other fruits were also found to contain citric acid. Pome and drupe fruits as a rule were found to contain malic acid.

As a product of the metabolism of fungi citric acid has been reported upon extensively by several authors. Wehmer ('93) reported on the genus *Citromyces* as containing acid producers. He obtained good yields of citric acid in the presence of $CaCO_3$. Martin ('16), using various species of the genus *Citromyces*, attempted to work out a commercially practicable method for producing citric acid by the fermentation of sugar but obtained insufficient yields. Butkewitsch ('22) studied quantitatively the

production of citric acid by *Citromyces glaber* and other species of this genus. Thom and Currie ('16) found citric acid to be produced transiently by various species of *Aspergillus*. Currie ('17), working with cultures of *Aspergillus*, was able to obtain good yields of citric acid, although Martin had previously discarded all such cultures as not producing this acid. The writer obtained citric acid from *Aspergillus* sp. and a *Penicillium* sp. on a dextrose medium.

Citric acid is not limited to the plant kingdom; it was early reported as a constituent of the milk of most mammals and of urine. Recently, quantitative studies have been carried out on the occurrence of citric acid in man. Amberg and McClure ('17) found it consistently present in urine and gave quantitative data for the amount excreted. Leake ('23) studied its occurrence in sweat under various conditions. Salant and Wise ('16) studied the physiological reaction of the animal body to varying doses of sodium citrate. While it is an excretion product in the human metabolism it actually occurs in very small quantities, however.

THE PHYSIOLOGICAL ROLE OF CITRIC ACID

As a source of carbon for fungi, citric acid, like most of the other organic acids, has received little attention. Nägeli ('80) listed it as second to tartaric acid as a source of carbon for the lower fungi. Waterman ('13) studied the use of citric acid and a number of other acids as compared with sugar, but the work on citric acid was not complete. He showed, however, that for *Aspergillus niger* this acid is a fair source of carbon. Currie ('17) suggested that citric acid might be one step in the course of the metabolism of sugar by *A. niger* and that it was used up as metabolism progressed, if the conditions were favorable. Butkewitsch ('22), working with *Citromyces glaber* and some *Penicillium*-like fungi, gave curves for the use of varying amounts of citric acid as well as for its production.

Numerous writers on pathological subjects have contributed notes on the "tolerance" of the various organic acids by fungi, but the conditions of acidity were usually not controlled and the limiting factors were more likely to be connected with the hydrogen-ion concentration than with the anion of the acid.

The work of Hemmi ('20) is a typical example of this latter type of work. Using a basic medium containing sugar and working with a large number of species of *Gloeosporium*, he studied the effect of the addition of varying amounts of organic acids. His general conclusion was that small amounts of these acids increased the growth of the fungi over that produced by the sugar alone, and larger amounts inhibited growth. The acids were added in the free state and no account taken of P_H so that the reasonable supposition would be that the depression of growth at the higher concentrations of acid was due to an unfavorable P_H. Unless the hydrogen-ion concentration is taken into account such studies give very uncertain results. The chief work of an analytical nature, therefore, has been done by workers using either *Aspergillus niger* or some of the fungi from the *Penicillium* group (including the fungi classified under the genus *Citromyces* Wehmer).

A number of earlier writers attempted to classify the products resulting from the fermentation of citric acid, and their results are outlined by Thiele ('11). From the results of these workers, using such inocula as spoiled cheese, hay decoction, etc., we find reported as fermentation products, butyric acid, acetic acid, succinic acid, ethyl alcohol, hydrogen, carbon dioxide, water, and carbonates. It is difficult to evaluate the results of the very early workers where neither culture methods nor chemical methods were well standardized, but the meager descriptions of the bulk of these experiments would point to yeasts and bacteria rather than true fungi as the organisms bringing about the fermentations. Special interest attaches itself, however, to the reported production of alcohols from citric acid, inasmuch as there was strong evidence in the present work that under certain conditions some of the fungi produced alcohols and acetic acid in the presence of citric acid, and presumably from it. Fairly recently Fitz ('78) reported alcoholic products obtained from the "spontaneous combustion" of calcium citrate. His tests gave isopropyl alcohol, a weak reaction for ethyl alcohol, and an uncertain test for succinic acid. Here again we know nothing of the actual agent of fermentation but it was probably bacterial. The difficulties in testing for such substances have no doubt hindered the work and affected the accuracy of the reports, for it is seldom that a botanist

is equipped to do careful work in organic chemistry. Currie ('17) stated that citric acid might yield oxalic acid in the course of metabolism, and my own work leads to the same conclusion, nor is it surprising that a fungus which normally produces oxalic acid from sugars should produce it from citric acid.

There seems to be a general agreement among bacteriologists that carbonates are formed by certain bacteria from the salts of organic acids and that this accounts for the increasing alkalinity of the solution. Maasen ('96) reported this finding for various bacteria and even went so far as to show how the citric and other acids were decomposed to form carbonates as end products. Ayers and Rupp ('18), Wolf ('22), and others confirm these findings. Wolf ('22) explained the reversal of reaction of *B. diphtheriae* cultures by the formation of alkali carbonates and gave figures for the amounts of CO_2 obtained from cultures of various ages as confirmatory proof. A critical examination of the data and the reactions involved would seem to indicate that this explanation might empirically delineate the situation, yet what actually happens is that the carbonates are formed as a result of the increasing alkalinity of the solution and as a consequence are a result and not a cause. Cultures of fungi were shown to produce an alkalinity as great as that produced by *B. diphtheriae* (see *Penicillium stoloniferum* and *P.* sp.) without carbonates being detectable in any appreciable amount. An inspection of the various equilibria involved may explain the situation.

H_2CO_3 dissociates in two stages according to the following two equations:

(1) $H_2CO_3 \rightleftarrows H^+ + HCO_3^-$

(2) $HCO_3 \rightleftarrows H^+ + CO_3^{--}$

Blasdale ('18) gives the dissociation constant (k) for the first equation as 3×10^{-7}, and for the second equation as 3×10^{-11}. However, H_2CO_3 is not stable in acid solution but decomposes to form H_2O and CO_2. An alkaline carbonate, such as Na_2CO_3, reacts according to the following equations when acid is added.

(3) $Na_2CO_3 + HCl \rightleftarrows NaCl + NaHCO_3$

(4) $NaHCO_3 + HCl \rightleftarrows NaCl + H_2CO_3$

(5) $H_2CO_3 \rightleftarrows H_2O + CO_2$

As the reaction proceeds to the right in equation (3) the true end point would be indicated by the dissociation constant for HCO_3 (equation (2)) and that for equation (4) by the dissociation constant for H_2CO_3, but H_2CO_3 instead of dissociating at a high acidity as H^+ and HCO_3^- decomposes to form H_2O and CO_2 and so does not accumulate in the solution as the free acid. Now if we reverse the scheme and pass CO_2 into a solution the amount of CO_2 retained by the solution is dependent entirely upon the acidity, both total and actual, of the solution. From the dissociation constant for HCO_3, 3×10^{-11}, we find that the P_H is about 5.3. On the acid side of this point the amount of CO_2 retained would be very small unless, according to Clark ('20), carbonates were used as buffers. On the alkaline side of 5.3, CO_2 would be absorbed in proportion to the total alkalinity present and carbonates would be formed (total alkalinity determined by titrating with an indicator that changes at P_H 5.3).

Consider now the situation when the salt of an organic acid such as citric acid is added to the solution and the solution left in an alkaline condition, assuming that the salt is sodium citrate. As the citrate radical is oxidized to CO_2, H_2O, etc., there is a consequent accumulation of Na ions in the solution, since the Na cannot be respired into the atmosphere and since it is exceedingly unlikely that it can all be absorbed into the organism and be neutralized. Consequently the assumption would be that in order to keep a proper degree of acidity in the protoplasmic mass it is excluded from entering in more than small quantities into the organism. The natural effect of this accumulation of the alkaline ions is the trend in the alkaline direction. Under these conditions the CO_2 respired by the bacteria is neutralized with the consequent formation of carbonates, the kind and amount depending directly on the free basic ions, the process being one of neutralization. There is certainly no valid reason for the idea of Maasen ('95) and others that carbonates *per se* are split off from the acid molecule; the obvious and direct explanation is that the CO_2 of respiration is neutralized by the basic ions resulting from the metabolism of the bacteria. Nor would it even be necessary for metallic ions to be present since some of the organic bases might

serve as well where the release of CO_2 is slow and takes place *in* the solution.

There is perhaps no ultimate difference between saying that a bacterium produces carbonates and finding carbonates formed by the neutralization of CO_2, yet there is considerable actual difference when the intimation goes with it that organic salts are "fermented" to form carbonates instead of CO_2 and H_2O. If we are to consider carbonates as products of metabolism then we must consider that H_2CO_3 and not CO_2 and H_2O are the products of respiration of most organisms and that it is only the environment which breaks up the acid into CO_2 and H_2O.

In considering the case of certain fungi using organic acids, we find these organisms growing in a medium too acid for the fixation of CO_2. As the course of active metabolism nears a close the solution rapidly becomes alkaline (see experimental results), yet not even traces of carbonates can be detected by ordinary methods. The reasons for this are probably two-fold. The fungus has used the free acid first, then the acid salts, leaving only the alkaline salts which, owing to the alkalinity of the solution, are metabolized with difficulty and the CO_2 given off by the fungous mat falls to a very low figure, and probably almost stops when even moderate alkalinity is reached. The CO_2 that continues to be given off is that resulting from the autolysis of the fungous mat but this is in large measure passed off into the air. So slowly is the CO_2 given off at this stage that the gradient through the stopper to the outer atmosphere is probably sufficient to prevent the accumulation of any considerable CO_2 tension in the atmosphere of the flask. Weakly alkaline solutions do not greedily absorb CO_2 from the air. Undoubtedly the use of a sensitive apparatus for the determination of small quantities of CO_2 and carbonates, such as that used in determinations with blood, would show considerable CO_2 evolved from these alkaline cultures upon the addition of acid in comparison with that which could be detected in the original solution. It is equally obvious that the amount of CO_2 present in the culture solution of a fungus which has ceased to consume the carbohydrate, due to the increasing alkalinity of the medium, will never equal the amount produced by bacteria growing *in* the solution and continuing active de-

struction of the acid anion, with the consequent release of basic cations to be neutralized. Instead of being the cause of increasing alkalinity, the neutralization of these basic ions by CO_2 is in all probability a means of keeping the solution from becoming even more alkaline than it ordinarily does.

The process of formation of intermediate metabolic products from citrates is very obscure. The formula of citric acid is sufficiently complex to permit of considerable adaptation to oxidation, and a few directions which such processes might follow will be indicated.

Citric and malic acids are closely allied in structure and reactions.

Citric acid

$$\begin{array}{l} CH_2.COOH \\ | \\ C(OH).COOH \\ | \\ CH_2.COOH \end{array}$$

Malic acid

$$\begin{array}{l} CH(OH).COOH \\ | \\ CH_2.COOH \end{array}$$

It is probable that the substituted OH group in these acids is the path of easy chemical access to the citrate and malate molecules. As was previously noted in connection with the oxidation of the various acids, succinic acid was not oxidizable either by permanganate or chromic acid, while malic (hydroxy-succinic) and tartaric (di-hydroxy-succinic) were readily oxidized by both substances, as is citric acid, which has a similar substitution. Likewise, benzoic acid and phenol are oxidizable with difficulty, whereas resorcin and phloroglucin are readily oxidized. Moreover, it is a general tenet of organic chemistry that compounds containing substituted hydroxy groups are less stable usually than the unsubstituted compound.

Sando and Bartlett ('21) pointed out that malic acid in extracted fruit juices breaks down spontaneously in the presence of toluol and chloroform to form succinic acid. Citric acid under the same conditions might be expected to form tri-carballylic acid. Both succinic and tri-carballylic acids, especially the

latter, are difficult to identify positively in small quantities. The formation of these 2 easily oxidized, hydroxy acids in such large quantities by plants, instead of the unsubstituted acids, is probably quite significant, and would class these compounds as storage products rather than as ultimate waste products.

It would be expected, in view of the ease with which citric acid is oxidized to acetonedicarbonic acid, that acetone might readily be a product of metabolism, That this may actually be the case in some instances was indicated by the fact that a distillate giving a profuse iodoform test in the cold was obtained from a culture solution of *Diplodia natalensis*.

The general indications are that CH_3COOH, C_2H_5OH, and other alcohols are produced under conditions where the O_2 supply is insufficient. The case of isopropyl alcohol and butyric acid is not well established, but it would seem probable that if one were formed the other might be formed also. However, such a splitting of the citrate molecule would apparently furnish little energy to the organism.

Miscellaneous Chemical Methods

TOTAL OR TITRATABLE ACIDITY

Sodium hydroxide and phenolphthalein were used for the titration of culture media. In part of the work the samples were aerated with CO_2-free air before titration, but comparison of aerated and unaerated samples showed so little difference that the procedure was abandoned. Titrations were carried to a strong pink color, since, according to Merck's handbook, both oxalic and citric acids are completely neutralized by this procedure. In so far as this method was applied to culture solutions it must also be noted that any KH_2PO_4 present in the solution would be titrated to K_2HPO_4.

In some of the work the amount of citrate was roughly estimated by titration with HCl, using thymol blue as indicator. Thymol blue changes from yellow to pinkish orange as the P_H changes from 1.8 to 2.0, but the end point is uncertain even when blanks of free citric acid and the indicator are used. This is especially the case when ammonium salts are present in the solution.

HYDROGEN-ION CONCENTRATION

The P_H of the solutions used was determined colorimetrically, using the Clark ('20) series of buffers and indicators.

REDUCING SUGARS

Dextrose was used throughout this work, and quantitative determinations were made by the Shaffer and Hartmann ('21) method, using the adaptation of Fehling's solution.

OXALIC ACID

For either the qualitative or quantitative determination of oxalic acid the precipitation with calcium acetate in a hot solution acidified with CH_3COOH as described by Leffmann ('17) was made use of. The precipitate from the acidified solution was dissolved in H_2SO_4 and tested qualitatively with $KMnO_4$ and MnO_2 or determined quantitatively by titration with standardized $KMnO_4$.

THE DETERMINATION OF TOTAL CARBON

It was found desirable to have a means for determining with rapidity the total carbon in the culture solutions used. This involved the oxidation of dextrose, citric acid (peptone in a few instances), and any metabolic derivatives of these substances. A number of dry combustion methods were examined, but besides being complicated and slow required too much equipment. A wet combustion method as sometimes used for carbon determinations in soil and steel seemed more feasible, while the method of Friedeman ('21) seemed to fill the need very satisfactorily. Since Friedeman's work has not been published, a brief account of the method will be included, together with data as to its application and accuracy in physiological work.[1]

Chemistry of the method.—Oxidation by chromic and sulphuric acids in the presence of H_3PO_4 is made use of in this method. The CO_2 formed when the reaction mixture is heated is aerated over into a modified Truog ('15) absorption tower and absorbed

[1] The writer is especially indebted to Mr. Friedeman for personal aid in the working up of the method and apparatus used, and for advice on the chemistry involved in its utilization in this line of work.

there with NaOH. In the original method $Ba(OH)_2$ was used to absorb the CO_2 but NaOH was found more satisfactory, being less difficult to handle in the air and forming no precipitate in the tower. $BaCl_2$ is added just before titration and the excess NaOH titrated in the presence of the precipitated carbonate according to the method of Bear and Salter ('16). The end point of the titration is very sharp but a little slow due to adsorption of the indicator by the particles of the precipitate. The difference between the titration of a blank on the chemicals and the titration of the determination represents CO_2, 1 cc. of 0.5 *N* HCl being equivalent to 3 mgms. of carbon. Concerning the technical chemistry of the method the reader is referred to Friedeman's work.

Apparatus.—A single unit of the apparatus is shown in fig. 1, and in pl. 14 is shown the equipment used, which included 4 such units. The figure and plate are largely self-explanatory. The tower (F) is made of glass tubing 25 mm. in internal diameter blown on to tubing of 5–6 mm. internal diameter. The length from "a" to "b" is 45–50 cm. and from "b" to "c" 30 cm. A perforated porcelain filter plate is placed at "b" and the tower filled about two-thirds full of glass beads (one 4-mm. perforated bead to two 3-mm. solid beads). When the joints are closed and suction applied liquid in E is drawn up over the beads in F. Carbon dioxide-free air is supplied by a soda-lime tower and the aeration tube for B should be of very small diameter, drawn down at the tip and bent as in fig. 1 to prevent the solution backing up into the tube during heating. The suction must be steady and a Richards pump was found very desirable; the bubbler G aids in keeping the suction steady, and, the addition of a drop of alkali and a drop of phenolphthalein indicates whether any CO_2 is being carried over from the absorption tower.

Reagents.—The following reagents are necessary: an oxidizing mixture, consisting of 340 gms. of chromic acid crystals (chromic trioxide) dissolved in 600 cc. of hot distilled water and diluted to 1 liter with syrupy H_3PO_4; syrupy H_3PO_4, 85 per cent, a good commercial grade; conc. H_2SO_4, 1.84 sp. gr., a good commercial grade; 0.5 *N* NaOH; 0.5 *N* HCl, standardized to be used for titration; $BaCl_2.2H_2O$, 75 gms. per 500 cc. of solution (rough

weight), 10 cc. of this solution being equivalent to 25 cc. of the 0.5 *N* NaOH.

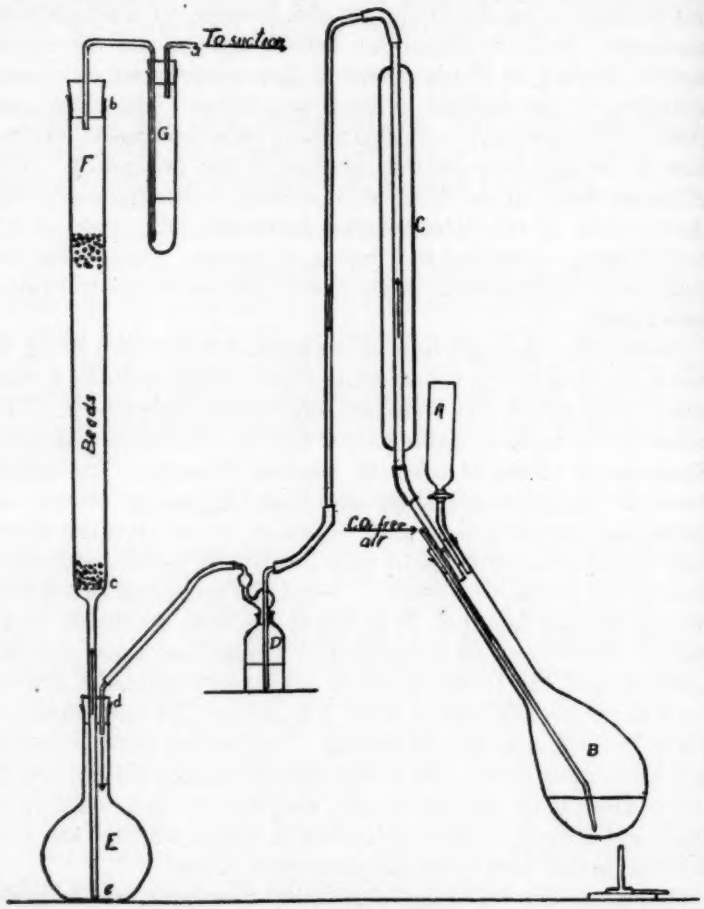


Fig. 1. Diagram of carbon analysis apparatus: *A*, separatory funnel; *B*, Kjeldahl flask; *C*, condenser; *D*, gas wash bottle; *E*, 500-cc. Florence flask; *F*, absorption tower; *G*, bubbler or trap.

Procedure.—Pipette into the Kjeldahl flask an amount of solution containing not more than 125 mgms. of carbon. Add enough distilled H_2O to make a volume of 50 cc. and a few glass

beads to prevent bumping. Attach the flask to the apparatus, make all the connections air-tight, and aerate briskly for 5 minutes to free the apparatus from CO_2. Close the pinch-cock at "a", loosen the rubber stopper at "d", and raise the tower enough to permit pipetting in 50 cc. of NaOH (25 cc. if only a small amount of carbon is present). Connect again and start aerating steadily. The solution automatically rises in the tower and covers the beads. Start the water running through the condensers. Add 10 cc. of the oxidizing solution through the separatory funnel A and follow it with 25 cc. of H_3PO_4 and 25 cc. of H_2SO_4. Heat cautiously with a low flame until the reaction mixture boils, to avoid forcing the solution back into the aeration tube. Continue boiling and aeration for 30 minutes, cut off the heat, aerate briskly for 5 minutes, then cut off the suction and raise the tower and fix it in a clamp so that it drains into the flask E. Wash the tower with small quantities of distilled water, using a total of 250 cc. and allowing a few seconds for draining after each addition. This method of washing removes practically all the NaOH to the flask E, it being less difficult to free the tower of alkali when NaOH is used than when $Ba(OH)_2$, as in the original method, since there is no precipitate of $BaCO_3$ to hold back the solution. Add 20 cc. of the $BaCl_2$ solution and 1 cc. phenolphthalein and titrate. The difference between the titration of the determination and that of a blank gives the amount of HCl equivalent to carbon.

Efficiency of the method.—Both Friedeman ('21) and Schollenberger ('16) gave data to indicate that this method of oxidation as applied to soils gave results approximating very closely those given by the usual dry combustion methods. Friedeman's ('21) figures on the combustion of cane sugar by his method indicated about 98 per cent oxidation as compared with figures by the dry combustion method. The writer was not equipped to carry out dry combustions but combustions by the Friedeman method were carried out on a number of solutions made up by weight as accurately as possible and then checked by other methods. The figures for the percentage of oxidation on duplicate determinations were as follows:

Dextrose	100 per cent
Citric acid	99.8 per cent
Oxalic acid	98.1 per cent
Malic acid	97.5 per cent
Tartaric acid	99.3 per cent

The above figures are only indicative of the general efficiency of the method, since it is very difficult to obtain some of the above acids in a pure state or to prepare accurately known solutions by ordinary methods. Some of the readily volatile substances, such as acetic acid and the lighter alcohols, are not oxidized by this method, and whether this is due to their volatilization or to their natural resistance to such an oxidation is undecided. Succinic acid is likewise unoxidized, possibly due to the formation of the stable succinic anhydride. It will be found, however, that the common fruit acids and sugars yield results of a good grade of accuracy by this method.

Experimental Work

The fungi employed

In carrying out the physiological work reported here a considerable number of fungi was used. Some of the cultures proved to be poorly adapted to culture work or too irregular in growth on artificial media and had to be abandoned, e. g., *Pythiacystis citrophthora* which failed to grow consistently in any liquid medium, and *Oospora Citri-aurantii* which was found very interesting but impracticable to use, due to the fact that no mat is formed, the growth breaking up to form a fine sediment. Some of the cultures were not obtained until the work was well under way and others failed to respond satisfactorily at various times; thus, in numerous phases of the work complete data on some of the fungi are lacking. A satisfactory culture of *Penicillium italicum* was never obtained, the culture used early in the work being so attenuated that it refused to infect oranges, and another used for a time as *P. italicum* proving later to be a different species (so identified by Dr. Thom). Nor was *P. italicum* found on rotting fruit in the St. Louis market during the winter. This was a disappointment since this fungus is un-

doubtedly an important one in the rotting of citrus fruits. The examination of a considerable amount of decayed fruit in shipments from Florida and California indicated that this fungus may not be as common in the winter as at other times, and that there are other fungi of this group with blue-green or green spores which may be at times mistaken for it. The organisms used in the work are reported upon below:

Penicillium stoloniferum Thom.—This fungus was sent by Dr. Fawcett of the Citrus Experiment Station of California. It had been isolated from rotting citrus fruit and was thought to be *P. digitatum* at the time, but it was later identified by Dr. Thom as *P. stoloniferum*. The habitat given by Thom ('10) is decaying Polypores and Boleti, but the fungus was found by the writer on decaying masses of citrus fruits. The culture was found infective to a certain degree, and it readily attacked lemons or oranges which had been partially rotted by other fungi. It probably does not constitute a primary agent in the infection of these fruits under ordinary conditions, but is of secondary importance, bringing about a final destructive rot. It grew well on most synthetic media, tolerated high acidity, used citric acid readily, and usually produced an alkaline reaction in the medium. The spores are considerably smaller than those of *P. digitatum* and the spore masses do not have the same olive-green color.

Penicillium sp.—This fungus together with the one just discussed, was sent to Dr. Thom, for it had been thought that this might be *P. italicum*, although the spores were considerably smaller than those described for that fungus. Dr. Thom stated that it was not related to *P. italicum* and that he could not name it definitely. Its growth on most media was blue-green to gray and very vigorous. It grew well on synthetic media, used citric acid readily, and usually produced an alkaline reaction in the culture medium. In general its reactions were very similar to those of *P. stoloniferum*.

Penicillium digitatum Sacc.—This fungus is well known as the cause of a destructive rot of lemons often seen in the market. It was a poor organism for cultural work since it did not grow well unless peptone was supplied as a source of nitrogen. It used citric acid, but in a fermentative way, since there was no

weight increase when this reagent was added to the medium. It is one of the commonest of the citrus fruit-rotting fungi on the market and can be easily distinguished by the olive-green color and large size of the spores.

Aspergillus sp.—This fungus was reported by Dr. Fawcett as rotting fruit at 27°, 30°, and 34° C. In spore size and the structure of the spore-bearing heads it corresponds with *A. niger*, but the spores in mass appeared first cinnamon-brown and then dark brown, and rarely if ever were dark enough to be considered black. It grew readily in most media, using the ordinary inorganic nitrogen sources. It also produced acid under certain conditions but apparently not so abundantly as the true *A. niger*. It may be a strain of *A. niger* or of a closely related species.

Diplodia natalensis Evans.—Cultures of this fungus were also furnished by Dr. Fawcett. The fungus grew well on most of the common synthetic media, forming a black, carbonaceous mat of close texture, but it did not form pycnidia readily. After growth the culture solution was dark colored, a deep red as the fungus matured, but if citric acid salts had been added this color was lessened. This fungus was originally reported from South Africa, where it caused a black rot of citrus fruits, and Fawcett ('15) later reported the fungus from Cuba, on grapefruit.

Alternaria Citri Pierce.—This fungus was also furnished by Dr. Fawcett. It was a slow-grower on most media, taking more than 20 days to come to a maximum growth. It produced spores very sparsely and did not grow at a high acidity. This fungus was originally described by Pierce ('02) as the cause of black rot of navel oranges, but the description was short and the inoculation data incomplete. Later, Patterson, Charles, and Veihmeyer ('10) reported a *Stemphylium* isolated from oranges affected with black rot. No further work has been reported in connection with the pathogenicity of this organism. This fungus will be contrasted with the next one mentioned, in regard to cultural characters.

Alternaria sp.—On the local market I found lemons apparently rotting with a typical brown rot, but in attempting to isolate *Pythiacystis* from these an *Alternaria* was frequently found. Various cultures of this organism were obtained and compared with

Alternaria Citri Pierce. The cultural characters were not greatly different, and the differences might well be interpreted as due to the development of the fungus in a different environment.

The spores of this *Alternaria* were consistently larger than those from the culture of *Alternaria Citri* and yet the spore sizes of both fell inside the limits prescribed by Pierce ('02). A brief comparison of the cultural characteristics of these two cultures is given below and checked against the description by Pierce ('02).

Spores.—The spores of the culture of *A. Citri* used were few in number, and within the limits of 11–22 × 5.5–8.2 μ in a large number of spores measured from various transfers. The spores exhibited 1–3 cross-walls, were light brown to dark brown, and in chains of 2 or 3. Germination was slow and the percentage of germination low. The spores of *Alternaria* sp. were numerous and within the limits 23.3–35.7 × 8.2–13.2 μ. There were 1–7 cells divided off by longitudinal as well as transverse walls, dark brown in color, and when viewed in position in a Petri dish culture were seen to be in long branching chains. These spores germinated readily in 24 hours at P$_H$ 3.0, in 31 hours at P$_H$ 2.7, and a few germinated at P$_H$ 2.5. Pierce ('02) gave the dimensions of spores as 10–22 × 8–15 to 25–40 × 15–25 μ, 3–6-septate, dark olive-brown, and 3–6-catenulate. These limits would include both of the cultures used by the writer.

Cultural characteristics.—*A. Citri* was a slow-growing species, not doing well on inorganic nitrogen sources. On solid media (agar) it formed a nearly circular colony showing marked radiate growth with little or no aerial mycelium. The colony appeared black on both sides except for the white, growing edge. In liquid media the mat was white, growing *in* the solution. *Alternaria* sp. was a profuse grower as compared with the *A. Citri* culture used. On agar plates the culture was black when viewed on the reverse side, with a narrow white edge, and it was decidedly zonate. The upper surface was covered with white to gray aerial mycelium. Under such conditions spores were sometimes produced profusely and at other times almost not at all. The amount of aerial growth was likewise irregular, weather conditions, i. e., temperature and humidity, apparently being the deciding factors. Older cultures produced fewer spores, indicating that the failure of the *A. Citri* culture might be due to too long a period in culture. In liquid media the mat tended to become pinkish, where there was a good supply of carbohydrate, and later dark. The mat grew *in* the solution.

Pathogenicity.—When inoculated into ripe, sterile lemons *A. Citri* produced, in some instances, a small rotted area limited to the rag, but in some of these rotted areas there were signs of contamination. On inoculation, by cutting into the rind and inserting mycelium, *Alternaria* sp. produced a definite rot limited largely to the rag and

carpel walls which were completely digested. On the surface there was a dark central area surrounded by a brown area shading to pink at the outer edges. This fungus also attacked the damp cotton upon which the sterilized lemons had been placed and digested it rapidly in spite of the fact that it had only been wet with sterile, distilled water.

Cultures of these fungi were sent to Mrs. Patterson, of the Office of Pathological Collections, Bureau of Plant Industry, and examined there by Miss Jenkins, who was of the opinion that they might represent different species. Subsequently it was learned that the various California species of *Alternaria* were being studied at the Citrus Experiment Station, Riverside, so no further identification work was carried out.

Phomopsis Citri Fawcett.—This culture was furnished by Dr. Fawcett, while later cultures were easily isolated from rotting oranges from Florida. The fungus did not grow well in most liquid media as it grew *in* the solution instead of forming a mat on the surface. Due to this habit of growth, it probably obtained insufficient oxygen in liquid culture, this being indicated by the results of some of the culture work. This fungus is the cause of a large amount of the rotting of oranges shipped from the Florida district. It did not grow at high acidity nor did it rot lemons.

Sclerotinia Libertiana Fuckel.—Sclerotia of this organism were obtained from Professor Horne of the University of California Agricultural Experiment Station. The sclerotia germinated readily, but this fungus was found to be very erratic in culture. In the presence of peptone it usually grew well in liquid media, but with inorganic nitrogen the growth tended to be scant and the formation of sclerotia occurred very early in the period of growth. This is the cause of the so-called "cottony rot" of lemons which occurs chiefly in the packed crates of fruit.

In order to facilitate the making of tables and the discussion of results the organisms used will usually be referred to by number as follows:

2. *Penicillium stoloniferum*	7. *Alternaria Citri*
3. *Penicillium* sp.	9. *Aspergillus* sp.
4. *Diplodia natalensis*	11. *Sclerotinia Libertiana*
5. *Phomopsis Citri*	14. *Alternaria* sp.
6. *Oospora Citri-aurantii*	16. *Penicillium digitatum.*

Cultural methods.—In carrying out the succeeding work the

culture solution used, with one exception, was based on a solution developed at this laboratory by Dr. Duggar.[1] The final concentrations of chemicals were as follows: M/4 dextrose, M/5 KNO_3, M/20 KH_2PO_4, M/100 $MgSO_4$ and a trace of $FePO_4$. For obtaining these dilutions the following stock solutions were used: M/2 dextrose, M/1 KNO_3, M/4 KH_2PO_4, M/10 $MgSO_4$, and M/1000 $FePO_4$. For 50 cc. of medium the following amounts of these solutions were used: 25 cc. dextrose, 10 cc. KNO_3, 10 cc. KH_2PO_4, 5 cc. $MgSO_4$, and 6 drops $FePO_4$. In some instances M/1 NH_4NO_3 or a peptone solution containing 8.8 gms. per liter and considered as M/1 for nitrogen was used instead of the KNO_3 solution. Two sizes of flasks were used, 300 cc. and 100 cc., 50 cc. of medium being used in the larger and 25 cc. in the smaller. Pyrex flasks, frequently cleaned with chromic acid cleaning solution, were used almost entirely. Care was taken at all times to have the glassware scrupulously clean.

Inoculations were made into liquid culture media by the use of spore suspensions where spores were produced. Where no spores were produced the fungus was grown in plate culture on potato agar, and squares about 3 mm. in dimensions, cut around the periphery of the colony, were used for inoculation. In one series of experiments the cultures were kept at 20° and 30° C. but for the most part they were kept at 25° C. The latter temperature is probably very near the optimum for most of the fungi used, and it seemed the most suitable temperature as far as the entire group was concerned. For determining the amount of growth, the fungous mat was filtered off on a filter-paper which had been dried to a constant weight at 103° C., weighed, and labelled. The mats were weighed after a similar drying. Weights were determined to milligrams as rapidly as possible to prevent absorption of moisture while on the balance.

The relation of acidity to growth.—In order to gain an idea of the limiting hydrogen-ion concentration for the growth of the fungi used some germination and growth tests were made. In a preliminary way spores of *Penicillium stoloniferum* and *P.* sp. were tried in a citric acid solution containing no other nutrients.

[1] A paper embracing the work from which this was taken will appear in a subsequent number of the Annals.

Spores were placed in hanging drops in Van Tieghem cells, using 1, 5, and 10 per cent citric acid and checks of distilled water. In 48 hours the distilled water controls of the *Penicillium* sp. had germinated, while the *P. stoloniferum* spores had germinated strongly and showed growth and branching in the 1 per cent citric acid and only slight germination in the distilled water. In the 5 and 10 per cent citric acid none of the spores of either fungus germinated. Previous experience had indicated that these 2 fungi were probably the only ones which would germinate in free citric acid.

For further study 2 solutions were made up as follows:

Solution I.—

M/1 KNO_3 ..... 100 cc.
M/4 KH_2PO_4 ..... 100 cc.
M/10 $MgSO_4$ ..... 50 cc.
Citric acid ..... 141 gms.
Dextrose ..... 20 gms.
M/1000 $FePO_4$ ..... 8 cc.
H_2O (distilled) to make 1 liter.

Solution II.—

KOH, 112.5 gms. per liter.

Solution I was approximately 2 *N* acid, and Solution II was 2 *N* alkali and consequently suitable for adjusting the reaction of Solution I. A titration curve was constructed by placing 5 cc. of Solution I in each of several test-tubes and adding varying amounts of Solution II and enough distilled water to make a total volume of 10 cc. and determining the P_H of the solution. By this procedure Solution I, which furnished all the nutrients, was diluted one-half. The results of this procedure are as follows:

Alkali added (cc.)	0.0	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.1	1.2	1.4	1.5	2.0	2.5
P_H	1.7	2.2	2.4	2.5	2.6	2.7	2.8	2.9	3.0	3.1	3.2	3.3	3.8	4.1

Autoclaving these solutions failed to change the P_H appreciably. Proceeding with these data, solutions of varying P_H were made up for use in germination and growth tests.

The germination of the spores of some of the fungi was tried by means of the hanging-drop method, following the general

procedure of Webb ('21) and the solution just described. Solutions with a P_H of 3.0, 2.7, 2.5, and 2.2 were tried with checks of sterilized, distilled water. One slide with 2 rings was used for the control and 3 slides with 2 rings each for the solution. The spores were mixed with a glass rod in a few drops of the solution on a clean slide until a suspension of proper strength had been attained and then were transferred to the cover slip. All slides were incubated at 25° C. Where possible 3 counts were made of each drop and the results averaged; in some cases the germination was so heavy and rapid that it could only be estimated. A few notes were made concerning the nature of the germination where it seemed to be especially significant. The results of these tests are given in table III.

TABLE III

PERCENTAGE OF GERMINATION AT P_H 3.0, 2.7, AND 2.5

Culture	Period (hours)	H-ion concentration			Check
		3.0	2.7	2.5	
14	24	24, 25, 35	44, 36, 25	None	67, 77
7	24	None	None		Few
9	24	90 (av.)	57, 40, 95	50*	100
9	48			Drops overgrown	
2	24	44, 75	———	———	0, 71
2	36		90, 50, 75	———	
3	24	87, 1, 25	Occasional	———	23
3	48		25, 50, 25	Occasional	25

* 50 per cent where spores were bunched, lower elsewhere.

Culture 9 gave such strong germination that it was difficult to estimate the percentage, the germination being much better where the spores were grouped together. At P_H 2.5 about 50 per cent of the bunched spores had germinated in 24 hours, while practically none of the isolated spores had germinated. This may have been due to the collective action of the bunched spores on the P_H of the surrounding solution. The results were extremely irregular, especially with the *Penicillium* spp. One circle might give almost 100 per cent germination and the other circle on the same slide little or no germination. For P_H 2.2 a tube method

was used, since it was felt that the hanging-drop method was too uncertain and might not give an actual indication of the ability of the fungi to form a mat at any certain hydrogen-ion concentration. Likewise this tube method offered a means for studying those fungi which did not form spores.

Solutions were made up to P_H 3.0 and 2.7 and 5-cc. amounts pipetted into 6-inch test-tubes and autoclaved. Three tubes at each P_H were inoculated with each of the following fungi: Nos. 5, 6, 7, 11, 14, and 4, the inoculations, with the exception of *Oospora*, being made from agar plates. The tubes were slanted to allow more surface for development and were then incubated at 25° C. The results after incubation of 10 days are given in table IV.

TABLE IV

GROWTH IN TUBE CULTURES AT P_H 3.0 AND 2.7

No.	P_H 3.0	P_H 2.7
4	Beginning	Beginning, 2 tubes
5	Beginning	No growth
6	Good growth	Good growth
7	No growth	No growth
11	Good growth	Good growth
14	Beginning	No growth

The above procedure was repeated at P_H 2.5 and 2.2 and the results are given in table V.

TABLE V

GROWTH IN TUBE CULTURES AT P_H 2.5 AND 2.2

No.	P_H 2.5	P_H 2.2
2	Good mat	Good mat
3	Good mat	Good mat
6	Clouding	Clouding
9	Good mat	Good mat
11	Beginning	Beginning
16	Beginning, 1 tube	No growth

The foregoing data would indicate that only 3 of the cultures were capable of growing readily in a medium as acid as lemon juice and with an inorganic source of nitrogen. In the case of

Sclerotinia the situation is a little more uncertain but the fungus would probably make some growth at this acidity. It was probable also that the source of nitrogen might make some difference and that when an organic nitrogenous compound, such as a protein, was available growth might occur at higher acidity. For this reason the reaction of the fungi to extracted orange and lemon juice was tried.

Oranges were peeled, the juice pressed from the pulp, the pulp wet with distilled water and pressed again, and this pressing added to the first. Six hundred and fifty cc. of juice were extracted from 10 oranges in this way, filtered through cotton, and pipetted in 25-cc. amounts into 120-cc. flasks. The P_H of the juice was 3.8. All the fungi used grew well on this juice. The extracted pulp was put in flasks after being washed until tasteless, a little distilled water added and autoclaved. The P_H of the last washings was 4.4. These flasks were inoculated and the fungi grew exceedingly well. The rind was minced up and put in flasks with a little distilled water and sterilized. Organisms 2, 3, and 7 grew slowly but eventually covered the rind completely, slowly dissolving the rag. Numbers 4, 5, 6, and 9 grew very rapidly, quickly covered all the pieces, and brought on a destructive, decomposition.

A lemon-juice extract was made as with the oranges, and for one set of flasks the juice was diluted with an equal amount of water (Solution I), the P_H being 2.5. For a second set the juice was diluted with an equal volume of distilled water to which had been added 25 gms. of Bacto dextrose per 300 cc. (Solution II), the P_H being 2.5. For a third batch, 100 cc. of 0.48 *N* KOH was added to 300 cc. of the diluted juice, making the acidity P_H 3.9 (Solution III). These solutions were pipetted into 120-cc. flasks, 25 cc. per flask, sterilized, and inoculated with organisms 2, 3, 4, 5, 6, 7, and 9. At the end of 10 days these were taken down, and the titer and the weight of the mat determined. For the results see table VI.

From the culture work reported above the fungi would seem to fall roughly into two groups: those that grow fairly well at a comparatively high acidity (P_H 2.0–3.0), comprising organisms 2, 3, 6, 9, and possibly 11; and a group of those not growing at

such high acidity, comprising organisms 4, 5, 7, 14, and 16. Of these 2 groups of fungi, Nos. 4, 11, and 14 varied considerably and were almost intermediate between the 2 groups. In the case of culture 4 the nitrogen source would seem to be of considerable importance in determining the limiting acidity. This

TABLE VI

GROWTH OF FUNGI ON LEMON-JUICE DECOCTION

Organism	Solution no.	P_H	Cc. N/20 KOH per 10 cc.	Wgt. of mat (mgms.)
	I	2.4	32.0	
	II	2.4	34.9	
Check	III	3.9	28.0	
2	I	3.2	20.0	125
	II	2.6	28.3	164
	III	5.0	7.2	170
3	I	2.4	32.1	No growth
	II	2.4	34.0	79
	III	4.2	16.9	137
4	I	2.6	23.4	36
	II	2.6	22.6	160
	III	4.2	12.0	179
5	I	2.4	27.0	No growth
	II	2.4	27.8	No growth
	III	3.9	16.8	155
7	I	2.5	24.3	No growth
	II	2.4	30.7	No growth
	III	4.2	18.2	77
9	I	2.5	24.1	29
	II	2.4	25.5	54
	III	4.1	23.8	180

division constitutes one which could be made by separating the cultures according to parasitism. Organisms 2, 3, and 9 are little more than saprophytes and are fungi which are secondary in importance as far as the rotting of fruit is concerned. Such fungi as *Phomopsis Citri* and *Alternaria Citri*, however, are very specialized rot fungi, attacking practically uninjured fruit.

A series of experiments was run to determine the general relation of citric acid to metabolism. The first series was calculated to determine what fungi could profitably use citric acid without any other source of carbohydrate being present. The culture solution previously described was made up, but a mixture of citric acid and potassium citrate was substituted for the dextrose, 8 gms. of citric acid and 12.35 gms. of potassium citrate (equivalent to 8 gms. of citric acid) per 800 cc. of solution. This solution was used in 100-cc. flasks, and the cultures were in triplicate. The results after 25 days of incubation at 25° C. are given in table VII. The weights of mats as given represent averages of 3.

TABLE VII

GROWTH OF FUNGI WITH CITRATE AS SOLE SOURCE OF CARBON

No.	Wgt. of mat (mgms.)	Remarks
2	55	Solution light orange-yellow, spores plentiful, mat gray.
3	71	No spores, mycelium white to gray, solution yellow.
4	No growth	
6		Growth with white sediment and clouding.
7	21	Just beginning growth.
11	12	Very slight aerial growth.

A second solution was made up, using the mineral nutrients in 1/5 the usual concentration and with 1 gm. of dextrose per liter. No citric acid was added at the start, but 200 cc. of citric acid solution were made up, using 21 gms. of citric acid, and this was sterilized in 10-cc. amounts in test-tubes, to be added to the flasks after growth had begun. This amount of citric acid, on being added to the solution in the flasks, gave 35 cc. of 3 per cent citric acid solution. Four 100-cc. flasks were prepared for each of the fungi used. After incubation for 8 days at 25° C. all of the cultures were found to be showing definite growth, and 2 of each 4 flasks inoculated with a fungus were removed to the transfer room and a tube of the citric acid solution added to each under sterile conditions. They were then returned to the incubator after agitation to mix the citric acid with the rest of the

solution. At the end of 25 days the weights of the mats were determined, and these results are given in table VIII. The weights for mats in both the blanks and the solutions to which citric acid was added represent the average of 2 mats.

TABLE VIII

GROWTH OF FUNGI WITH FREE CITRIC ACID

No.	Blank		Solution plus citric acid	
	Mat (mgms.)	Remarks	Mat (mgms.)	Remarks
2	19	Solution orange-yellow, spores grayish	167	Growth heavy, spore masses greenish
3	31	Little growth	201	Spores green, much white aerial mycelium
4	36	Complete mat formed	32	Incomplete mat
5	14		27	All growth *in* solution
7	22		32	
11	17	Solution black, some aerial growth	42	No aerial growth

The effect of nitrogen source on utilization of citric acid.—In order to test the effect of various nitrogen sources on the utilization of citric acid a stock solution was made up as follows: citric acid, 157.56 gms.; potassium citrate, 162.17 gms.; dextrose, 12.5 gms.; M/1000 $FePO_4$, 4.0 cc.; and distilled H_2O to make 1 liter.

Five cc. of the above stock solution diluted to 25 cc. gave a nutrient solution containing M/4 of the citrate and dextrose at the rate of 2.5 gms per liter. This solution had a P_H of 4.2. It was used as the source of carbon in the regular culture solution, the source of nitrogen being varied, KNO_3, NH_4NO_3, and peptone being used. It was found impossible to use $Ca(NO_3)_2$ satisfactorily due to the fact that on sterilization calcium citrate precipitated out, or if the nitrate was not added until after sterilization, as soon as the fungus grew a little the citrate began to precipitate out due to the change in P_H. The mineral nutrients were used in 2 concentrations, as originally given and 1/5 of that concentration. The amount of the solution furnishing the carbon was kept constant throughout. Blanks were run on the amount

of sugar used but without the citric acid, using KNO_3 as nitrogen source. The fungi were grown in triplicate in 100-cc. flasks, and the results are found in table IX, the weights of the mats representing averages of 3.

TABLE IX

GROWTH OF FUNGI ON CITRATE WITH VARIOUS N SOURCES

Cult. No.	Mineral nutrients, regular conc.				Mineral nutrients, $\frac{1}{4}$ conc.				Days
	KNO_3	NH_4NO_3	Peptone	Blank	KNO_3	NH_4NO_3	Peptone	Blank	
2	201	189	307	7	167	199	225		20
3	171	258	370	52	209	208	213		20
16	91	130	162	29	47	111	168		33
4	280	353	351	60	297	347	325	22	22
11	210	244	296	58	160	171	155	15	22
7	188	149	343	42	210	134	273	13	45
5	62	68	61	32	44	75	151	14	28
9	322	301	443	37	280	275	295	16	20

From these experiments we may draw certain general conclusions to be used in future culture work. None of the fungi would make any rapid or luxuriant growth with the citrate ion as the sole source of carbon but some would utilize it if a little sugar was allowed at the start. Organisms 2, 3, 9, and probably 6, grew very well on the free citric acid, unneutralized, after they were once started with a small amount of sugar (1 gm. per liter was sufficient). Organisms 4, 5, 7, 11, and probably 16 (judging from later work), would not make more than slight use of unneutralized citric acid even after being given a good start with sugar, the free citric acid being probably lethal to the mycelium. In most cases peptone was the best source of nitrogen in connection with the citrate radical, although there was no considerable advantage over KNO_3 or NH_4NO_3 in many cases, and with *Diplodia* NH_4NO_3 would seem to make a more favorable nitrogen source than either peptone or KNO_3. In interpreting the results where various sources of nitrogen were used it is to be noted that *Phomopsis Citri* and *Penicillium digitatum* showed very little use of citric acid and that *Alternaria Citri* showed little use in the presence of KNO_3 and NH_4NO_3. None of these results are to be taken as final in any sense. No culture work which only

accounts for growth over certain fixed periods is absolutely comparative—only growth curves plotted from frequent determinations can be truly comparative.

Use of varying amounts of citric acid.—Using peptone as a source of nitrogen a large series was run, using varying percentages of citric acid-potassium citrate mixture. The solution used was based on analyses of oranges and lemons given by various authors. Using M/10 $MgSO_4$ and M/5 KH_2PO_4, the following solution was used: KH_2PO_4, 300 cc.; $MgSO_4$, 30 cc.; peptone, 30 gms.; dextrose, 15 gms.; M/1000 $FePO_4$, trace; distilled H_2O to make 1 liter. This was diluted to make 1½ liters, this dilution allowing for the addition of the citric acid mixture. The citric acid solution contained 0.25 gm. per cc. and the P_H was adjusted by the use of a solution of potassium citrate equivalent to the acid solution in citrate ion. Using varying amounts of the citric acid and potassium citrate solution, titration curves were made for solutions containing 2½ and 5 per cent of the citrate radical; and another series of curves was constructed for the titration of the culture solution with citric and hydrochloric acids. From these data solutions were made up to P_H 3.0 and 4.5, using 3 different concentrations of acid, that is, 2½ and 5 per cent citric acid (citrate radical), just sufficient citric acid to obtain the desired P_H, and a check solution with just enough HCl to obtain the desired hydrogen-ion concentration. The fungi were grown in triplicate in 2 temperatures, that is, 18–20° C. and 30° C. At the end of varying periods, depending on the speed with which the fungus grew, the weights of the mats and the P_H of the culture solution were determined. The concentration of the dextrose was rather small (10 gms. per liter), and the fungi were dependent chiefly upon the citrate-citric acid mixture for carbonaceous material. The results are given in table x, the weights representing averages of triplicate cultures.

In considering the results shown in table x certain factors in regard to the solution must be kept in mind. The weights for the HCl blanks probably do not represent the maximum weight attained, the maximum in most instances probably coming before the cultures were taken down. This loss of weight by autolysis was probably not very great in any case except in that of *Sclero-*

tinia, the mats for this fungus being left for a long period due to slow growth in the citrate solution. The increasing amounts

TABLE X

GROWTH OF FUNGI IN MGMS. ON VARYING PERCENTAGES OF CITRATE

Acidifying agent	20° C.				30° C.			
	P_H 3.0		P_H 4.5		P_H 3.0		P_H 4.5	
	P_H	Mat	P_H	Mat	P_H	Mat	P_H	Mat
Culture 2–12 days								
HCl	7.6	189	7.0	168	3.8	25	6.0	156
Cit. Ac.	6.9	218	7.3	203	3.4	—	4.5	—
2½% cit.	6.8	330	6.9	332	3.3	135	4.6	185
5% cit.	6.7	444	7.0	447	3.4	45	6.6	353
Culture 3–12 days								
HCl	7.3	225	7.2	231	3.9	—	6.9	178
Cit. Ac.	?	257	7.5	223	3.7	47	4.5	—
2½% cit.	?	393	8.1	365	3.0	—	4.7	78
5% cit.	5.7	544	7.9	475	3.1	59	4.7	162
Culture 9–12 days								
HCl	6.4	220	4.6	211	6.2	178	6.4	171
Cit. Ac.	5.7	359	5.7	224	6.5	235	6.4	210
2½% cit.	6.2	429	5.9	414	6.1	345	6.4	303
5% cit.	4.6	649	6.6	511	6.1	487	6.4	404
Culture 4–12 days								
HCl	4.0	210	7.2	305	7.5	256	7.6	246
Cit. Ac.	3.6	221	7.2	305	7.5	280	7.9	228
2½% cit.	3.3	92	5.0	316	4.6	303	7.4	328
5% cit.	3.2	—	4.7	329	3.0	—	7.4	396
Culture 7–18 days								
HCl	3.0	24	8.1	168	3.0	—	7.4	127
Cit. Ac.	3.4	16	7.0	215	3.0	—	8.1	170
2½% cit.	3.2	—	4.4	134	3.0	—	6.4	334
5% cit.	3.0	—	4.5	102	3.0	—	4.4	397
Culture 11–43 days								
HCl	6.6	90	6.0	99	4.4	157	4.6	33
Cit. Ac.	6.6	176	6.3	104	5.0	183	5.2	51
2½% cit.	6.3	212	6.6	193	4.5	171	5.1	128
5% cit.	3.1	300	4.4	146	3.2	—	4.8	288

of citrate radical in the solutions represent more than an increasing amount of carbohydrate material and increasing osmotic pressure;

in addition they represent an extremely strong buffer. Thus if the optimum for a fungus is P_H 4.5 and it is inoculated into a medium adjusted to P_H 3.0 the amount of buffering in the solution is likely to be a factor in the end result. On the other hand, if the H-ion concentration of the solution into which the fungus is inoculated is near the optimum of that for the fungus, then the highly buffered solution will maintain an optimum condition longer against the production of acid or alkali than will the unbuffered solution. In the case of organism No. 4 at P_H 3.0, the growth became less as the amount of citrate radical was increased, when incubated at 20° C., but at 30° C. the growth increased up to the 2½ per cent concentration, the fungus not growing at all in the 5 per cent concentration. That this was most likely a case of buffer action maintaining an unfavorable reaction is indicated by the final hydrogen-ion concentrations. At 30° C. this concentration varied from P_H 7.5 to P_H 3.0. At the former concentration the fungus grew, while at the latter it had not grown and the P_H was unchanged. That the amount of citrate radical is not in itself the factor that decides whether the fungus will or will not grow is shown at P_H 4.5 where the growth increased progressively with the amount of the citrate radical added. The results for several of the fungi indicated clearly that the ability of a fungus to grow at a certain P_H is not based entirely on the hydrogen-ion concentration and nutrients but that temperature and other environmental conditions are vital factors to be considered as well as the ease with which the P_H of the solution can be shifted. Organism No. 2 grew well in all of the solutions at 20° C. and about equally well at P_H 3.0 and 4.5, but at 30° C. it grew very little at P_H 3.0 and well at 4.5. Organisms 4 and 9 were the only ones that grew satisfactorily at 30° C.

The use of free citric acid.—Further data on the ability of the various fungi to utilize free citric acid were obtained by using a fermentative method. The fungi were grown in 300-cc. flasks until they had formed a substantial mat but had not yet reached the peak of growth. The solutions to be tried—a sugar solution and a citric acid solution—containing a small amount of mineral nutrients, were sterilized in 50-cc. amounts in 120-cc. flasks. After the mat was formed the solution was poured off under aseptic

conditions, 50 cc. of sterile distilled water were added to the mat, allowed to stand 5 minutes, then poured off and added to the culture solution first removed. The 50 cc. of the sterile solution in the 120-cc. flask was then poured into the 300-cc. flask with the culture. All manipulations were carried out as carefully as possible, and care was taken to wet the top of the mat as little as possible when adding the water and the new solution. Pyrex flasks were used for the mats so that the necks could be flamed freely to avoid contamination. Three solutions containing 3 different nitrogen compounds, KNO_3, NH_4NO_3, and $Ca(NO_3)_2$, were used for comparison. The P_H of the combined solution and washings was determined and the solution used for this determination returned to the flask and the titer of the solution determined, using N/10 NaOH and phenolphthalein.

The solution used for the growth of the fungi was the regular solution diluted, the following concentrations being used: dextrose, 25 gms.; KNO_3, 40 cc.; KH_2PO_4, 40 cc.; $MgSO_4$, 20 cc.; $FePO_4$, trace; H_2O to make 1 liter.

The sugar solution tried fermentatively contained: dextrose, 25 gms.; N source (KNO_3, NH_4NO_3, or $Ca(NO_3)_2$), 25 cc.; KH_2PO_4, 10 cc.; $MgSO_4$, 5 cc.; $FePO_4$, trace; H_2O to make 1 liter. This solution was adjusted to an acidity of P_H 2.5 with HCl before sterilization. The citric acid solution was the same as the sugar solution except that 25 gms. of citric acid were substituted for the dextrose. The schedule of changes to which each of the 3 mats of each fungus was subjected was as follows:

Mat 1. KNO_3*-dex. → KNO_3-cit. → $Ca(NO_3)_2$-dex. → $Ca(NO_3)_2$-cit.

Mat 2. NH_4-dex. → NH_4-cit. → KNO_3-dex. → KNO_3-cit.

Mat 3. —— → $Ca(NO_3)_2$-cit. → NH_4-dex. → NH_4-cit.

It must be remembered that there must always be a slight difference between the blank of the solution and the solution removed from the fungous mat, even if there has been no reaction on the part of the fungus, since it is manifestly impossible to wash the mat completely free of the solution without unduly exposing it to contamination.

* The solutions will be differentiated as far as the nitrogen source is concerned by indicating the cation of the compound used, i. e., "Ca-dex" for "$Ca(NO_3)_2$-dextrose."

The data concerning the solution in which the mats were grown is given in table XI. It will be noted that 1 flask of each 3 was permitted to grow an additional period of 6 days, the original inoculations all being made on the same date.

TABLE XI

HYDROGEN-ION CONCENTRATION AND TITER OF CULTURE SOLUTION AFTER GROWTH OF FUNGOUS MATS

Organism	P_H	Cc. N/10 NaOH	Period (days)
Blank	4.4	5.15	8*
4	6.5	2.35	8*
4	6.8	1.4	14
9	1.8	57.5	8
9	2.1	37.7	14
2	7.5	1.05	9
2	7.8	0.0	15
3	5.4	6.25	9
3	6.7	1.2	15
7	6.4	3.05	10
7	6.8	2.0	16
11	5.0	5.12	10
11	5.0	8.5	16

* Data for the shorter periods represent averages of two cultures, for the longer periods one culture.

The results of the fermentation of the sugar solutions are given in table XII. These solutions were run in 2 separate series. The first series included KNO_3 and NH_4NO_3 as nitrogen sources and the second series, which followed after the first citric acid series, contained all 3 nitrogen sources. The solutions are listed according to the cation of the nitrogen-containing compound.

The results obtained when using the citric acid solution are given in table XIII. The 9-day series was run after the first sugar series and the 5-day series after the second sugar series.

A survey of the data in tables XI, XII, and XIII shows considerable variation among the fungi used, and these variations indicate in some measure the kind of reaction favorable to such a fungus. *Diplodia* produced more acid from sugar in the presence of NH_4NO_3 than when KNO_3 and $Ca(NO_3)_2$ were used, but in the case of *Aspergillus* sp. the most acid was found when KNO_3 was used. In the presence of KNO_3 the final titration of organism

4 (table XII) was less than the titration of the blank, while in the case of organism 9 all titrations on sugar media were more than

TABLE XII

HYDROGEN-ION CONCENTRATION AND TITER OF DEXTROSE SOLUTION AFTER FERMENTATION BY VARIOUS FUNGI

Organism	Solution	P_H	Cc. N/10 NaOH	Period of fermentation (days)
Blank	Ca	2.5	4.0	
	K	2.5	4.0	
	NH_4	2.5	4.6	
	K	6.9	1.1	6
	NH_4	2.1	9.2	6
2	Ca	7.2	1.0	3
	K	6.7	0.4	3
	NH_4	2.4	4.5	3
	K	5.2	2.3	6
	NH_4	6.5	1.7	6
3	Ca	7.4	0.8	3
	K	7.2	0.3	3
	NH_4	4.4	3.2	3
	K	6.8	0.95	6
	NH_4	2.2	7.3	6
4	Ca	4.2	5.2	3
	K			
	NH_4	2.4	21.75	3
7	K	7.0	0.8	6
	NH_4	4.2	2.1	6
	K	2.1	13.5	6
	NH_4	2.9	8.9	6
9	Ca	2.7	6.6	3
	K	2.2	18.35	3
	NH_4	2.0	8.35	3
	K	6.0	1.1	6
	NH_4	2.1	10.3	6
11	Ca	6.8	0.7	3
	K			
	NH_4	2.2	11.1	3

the blank. With the exception of *Penicillium stoloniferum*, in the presence of NH_4NO_3, no titration of the culture solutions of

TABLE XIII

HYDROGEN-ION CONCENTRATION AND TITER OF CITRIC ACID SOLUTION AFTER FERMENTATION BY VARIOUS FUNGI

Organism	Solution	P_H	Cc. N/10 NaOH	Period of fermentation (days)
Blank	Ca		170.0	
	K		170.0	
	NH_4		171.0	
	Ca	8.2	2 drops HCl	9
	K	8.2	2 drops HCl	9
	NH_4	7.6	1.05	9
2	Ca	7.8	1.0	5
	K	7.9	0.9	5
	NH_4	7.5	1.1	5
	Ca	8.2	2 drops HCl	9
	K	7.9	0.25	9
	NH_4	8.0	1 drop HCl	9
3	Ca	7.4	1.3	5
	K	8.0	0.6	5
	NH_4	2.8	23.2	5
	Ca	2.4	136.8	9
	K	2.8	134.3	9
	NH_4	2.2	145.9	9
4	Ca	2.4	111.25	5
	NH_4	2.4	119.0	5
	Ca	2.5	150.1	9
	K	2.4	145.0	9
7	NH	2.4	136.75	9
	Ca*			
	Ca	4.2	0.9	9
	K	4.4	1.75	9
	NH_4	3.7	2.15	9
9	Ca	6.6	1.3	5
	K	3.8	5.9	5
	NH_4	3.4	3.3	5
	Ca	2.8	74.4	9
	K	5.8	9.7	9
11	NH_4	2.3	94.7	9
	Ca	3.6	40.3	5
	NH_4	2.6	29.4	5

* Solution not changed, no growth since last change.

a *Penicillium* or of *Alternaria Citri* was more than the blank. *Sclerotinia Libertiana* produced acid in the presence of NH_4NO_3 but not in the presence of the other nitrogen sources.

In utilizing free citric acid the 2 species of *Penicillium* and *Aspergillus* sp. were undoubtedly most efficient. On blanks of about 170 cc. of N/10 NaOH the readings for these fungi, even after the short period of 5 days, were in the neighborhood of 5 cc. of N/10 NaOH or less. It is difficult to decide whether the source of nitrogen had any effect on the destruction of the acid, the case of organism 3 over the 5-day period being the only one which gives any indications (NH_4NO_3 seemed in this case a little less efficient than the other nitrogen sources). *Alternaria Citri* failed to survive the treatment with citric acid. There was an indication that some acid was used in the 9-day period but the amount was so small that possibly the very thick and spongy mat may have held back sufficient acid in washing to account for the loss. In the case of *Sclerotinia Libertiana* and *Diplodia natalensis* the figures would indicate that there was greater use of citric acid in the second period of 5 days than in the first period of 9 days. This may be due to the increased growth of the mat, resulting in greater absorbing surface. It would hardly be safe to attribute it to "acclimatization" of the fungus to this acid environment, although this might be the case. It will be noted that in the course of changing the solutions the original solution was made up with KNO_3. The first change was to a sugar solution with NH_4NO_3 and KNO_3 as nitrogen sources, while at the next change the same nitrogen sources were retained for these 2 mats. In both cases these mats were apparently dead or nearly so when the NH_4NO_3-citric acid solution was removed, nor did the mat revive and show growth when the second sugar solution was added. Peculiarly enough, when the NH_4NO_3-citric acid mixture was added to another mat on the second round, that is, the mat which had received the $Ca(NO_3)_2$-citric acid mixture on the first round, the usage of citric acid was practically as good in the case of *Diplodia* as with the $Ca(NO_3)_2$-citric acid solution, and in the case of *Sclerotinia Libertiana* a little better. Just how such data could be properly interpreted is a question.

Having obtained from the foregoing experiments sufficient

data to give a general understanding of the reactions of the various fungi, it seemed desirable to round out the work with a careful analytical study of their reactions to citric acid. For such a study it was desirable to compare a solution which contained only a sugar as a source of carbon with one containing citric acid in addition to the sugar. Growth data covering fixed periods were considered inadequate for such a study and analyses of the culture solution were resorted to. Such analyses and the weights of the mats were taken at frequent periods during the course of growth and curves were plotted from the data so obtained. This method gave a complete outline for comparison of the 2 solutions and obviated the difficulties due to variations in the periods of growth in the 2 solutions.

The culture solution developed by Dr. Duggar and described previously in this article was utilized in this part of the work. Had there been time available for extended comparative work it is probable that a more satisfactory solution could have been found for any one of the fungi or perhaps for the entire group. However, the solution used appeared to be well adapted to the group of fungi as a whole. Wherever possible KNO_3 was used as a source of nitrogen, since it simplified the analytical work; some of the fungi, however, required peptone, and for *Diplodia* NH_4NO_3 was used. Dextrose was used as the source of carbon since it was easily determined quantitatively and had given good results previously. The citric acid used was Merck's "Reagent," and the KOH and potassium citrate Merck's "Highest Purity."

The question of what cation to use in the partial neutralization of the citric acid was a difficult one. Ammonium citrate gave growth with all the fungi as both a nitrogen and supplementary carbon source, when used with a small amount of dextrose, but the NH_4 radical complicated the solution unnecessarily and was probably not an efficient source of nitrogen for most of the fungi. Sodium would probably have been satisfactory in many instances but its exact status in relation to the growth of fungi is unsettled and, at least in some instances, it appears to be toxic. Calcium precipitates an insoluble salt with citric acid on heating, and even if $Ca(NO_3)_2$ is added to the solution after sterilization calcium citrate is precipitated out as soon as the fungus starts

growth. The determining factor in the latter precipitation is apparently the hydrogen-ion concentration of the solution. The use of potassium would seem to unbalance the solution by increasing disproportionately the amount of this cation, since KNO_3 and KH_2PO_4 were being used as inorganic nutrients. However, potassium had already been used successfully, and as the citric acid was to be only partially neutralized it seemed preferable to use potassium rather than a cation of unknown physiological reaction. In adding a large amount of citrate radical to the solution it was apparent that one solution was to have a somewhat higher osmotic pressure than the other. To compensate for such a disparity it would have been necessary either to cut down the dextrose in the solution to a small amount and to substitute sufficient citrate mixture to make up for the dextrose or to add to the dextrose solution enough of an inert buffer substance to be equivalent to the osmotic pressure of the citrate mixture. Both of these methods would involve numerous difficulties. If the first method were used the amounts of dextrose in the 2 solutions would be so widely different as to make difficult an accurate comparison of the growth in the 2 solutions even if the amount of the citric acid-potassium citrate mixture to be added could be accurately determined, and the second method is at present impossible owing to the fact that no absolutely inert (physiologically) buffer mixture for culture media is known.

Using the usual concentrations of mineral nutrients, one solution contained M/4 dextrose as a source of carbon and was designated as solution 1. This solution, where necessary, was adjusted with H_3PO_4. A second solution contained M/4 dextrose and M/4 *citrate radical* (a mixture of citric acid and potassium citrate) and was designated as solution 2. The adjustment of P_H was accomplished by varying the ratio of citric acid to potassium citrate. When only a small amount of potassium citrate was needed, neutralization with 2 *N* KOH sufficed, but where a larger amount was needed solid potassium citrate was added. The resulting solution probably contained free citric acid and a mixture of 3 potassium salts of citric acid, that is, mono-, di-, and tri-basic citrates. In so far as possible the solutions were made up in bulk and distributed to the 300-cc. flasks by means of a

50-cc. volumetric flask. This procedure insured the uniformity of the culture solution in the various flasks. All cultures were incubated at 25° C.

For each experiment with a single fungus enough flasks of solution were made up for 2 cultures to be used for each set of determinations. These determinations were made at first on each flask and the results averaged. Later the contents of the 2 flasks were mixed together and the analysis on the mixed solution taken as the average. The fungous mat was filtered off on a weighed and folded filter and washed with distilled water, the filtrate and washings being collected in a clean 200-cc. volumetric flask. The flask was then made up to the mark with distilled water and mixed. Where the mat was sufficiently coherent it was washed as much as possible in the flask before removal to the filter. The titer, P_H, reducing sugar, and total carbon were determined, and where time was available other tests were carried out. The determinations were started on the first day that appreciable growth was visible and followed up at intervals calculated to give a number of points on the curve where change was rapid and fewer where the change was slower.

Where a large amount of routine work is to be carried out, long tedious processes of analyses must be eliminated in favor of shorter and simpler procedures even at the expense of absolute accuracy. Moreover, in dealing with the growth of organisms the variations are likely to be so great as to nullify the accuracy of any single determination. In interpreting the results, likewise, it is far more desirable to have a considerable number of results indicating a continued difference than to have a single result indicating a single difference. No matter how accurate the latter might be, the variations are sufficient to make it no better than an even chance that the single result represents a variation from the normal condition. These considerations were kept in mind in interpreting the results of the experimental work, and conclusions have been drawn only from clear, consistent differences.

In plotting the curves the loss of carbonaceous matter from the solution was plotted rather than the experimental figures, that is, the successive determinations were subtracted from the blank, giving differences or losses, and these losses were plotted. These

curves compare better with the mat-weight curves, which may be run parallel to them on the same figure, and likewise represent very well the progress of the metabolic activities. On the ordinates are plotted weights in tenths of a gram and on the abscissae the time in days, the ordinates in case of the mat-weight curves representing the weight of the mat in grams. The curves represent the actual analytical work, and in order to reduce the amount of detailed material presented the tables from which these curves were plotted are omitted. Dextrose and citric acid are both plotted as carbon, dextrose on the basis of 39.978 per cent carbon, and citric acid as 34.272 per cent, these percentages being calculated from the molecular weights of anhydrous dextrose and citric acid with one molecule of water of crystallization. In the following curves besides mat weight will be found: (1) "loss of dextrose" (calculated as carbon) as determined by the Shaffer method; (2) "loss of carbon" calculated from the total carbon determinations; (3) "loss of carbon—loss of dextrose" calculated by subtracting the figures for curve (1) from the figures for curve (2) (theoretically if no oxidizable end products were formed from either dextrose or citrate, this curve would represent the loss of citrate—actually it probably roughly approximates it); and (4) a curve for loss of acidity calculated from the titrations with NaOH (the results of the titrations in N/10 NaOH were calculated as citric acid and the equivalency of carbon determined from the percentage of carbon in citric acid). This latter curve might involve several errors due to such factors as the absorption of titratable phosphates, the production of oxalic acid from the citric acid or the production of acidic substances from the dextrose, nor would it throw any light on such a situation as might be brought about by differential absorption of the anion and cation of the citrate, that is, if the anion were absorbed and the cation remained in the solution to neutralize free citric acid present in the solution the titrations would indicate a utilization of free citric acid rather than combined citrate radical.

Organism 3 (*Penicillium* sp.) and organism 9 (*Aspergillus* sp.) were used in the first series. The solutions were made up to P_H 2.5. In the curves given for these fungi each figure plotted represents the average of 2 different determinations made on separate flasks of the culture.

Penicillium sp.—In fig. 2 are given the data for solution 1 and in fig. 3 the data for solution 2. It is to be noted in connection with these curves that the time elapsing between inoculation and the beginning of chemical determinations was 5 days in the case of solution 1, but in the case of solution 2 it was 10 days. Taking

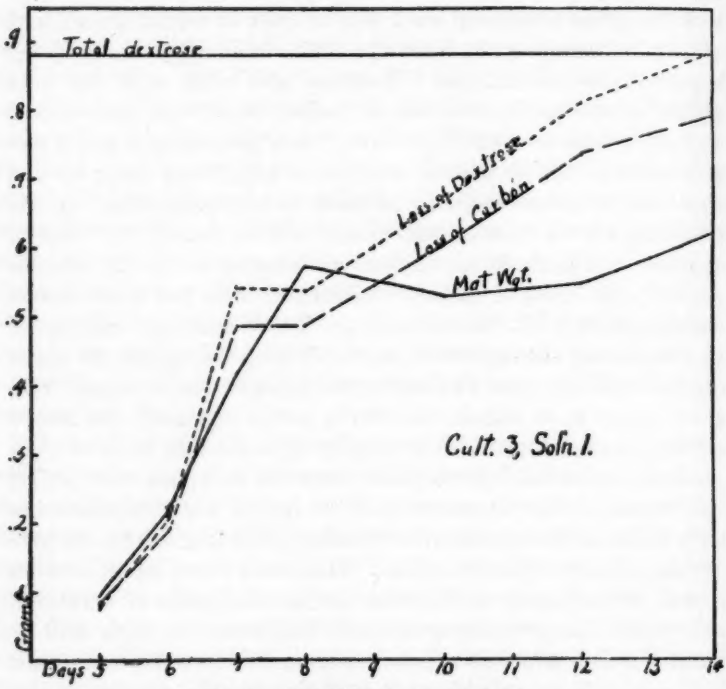


Fig. 2. *Penicillium* sp. in solution 1.

this into consideration there is to be noted a general deterrent effect upon the starting of growth in solution 2; this was probably due to the fact that the P_H of the solution was unfavorable for growth and that this situation was overcome much more readily in the slightly buffered sugar solution than in the heavily buffered solution 2.

The maximum weight of mat attained in solution 2 was not ar from twice that attained in solution 1. Apparently in solution

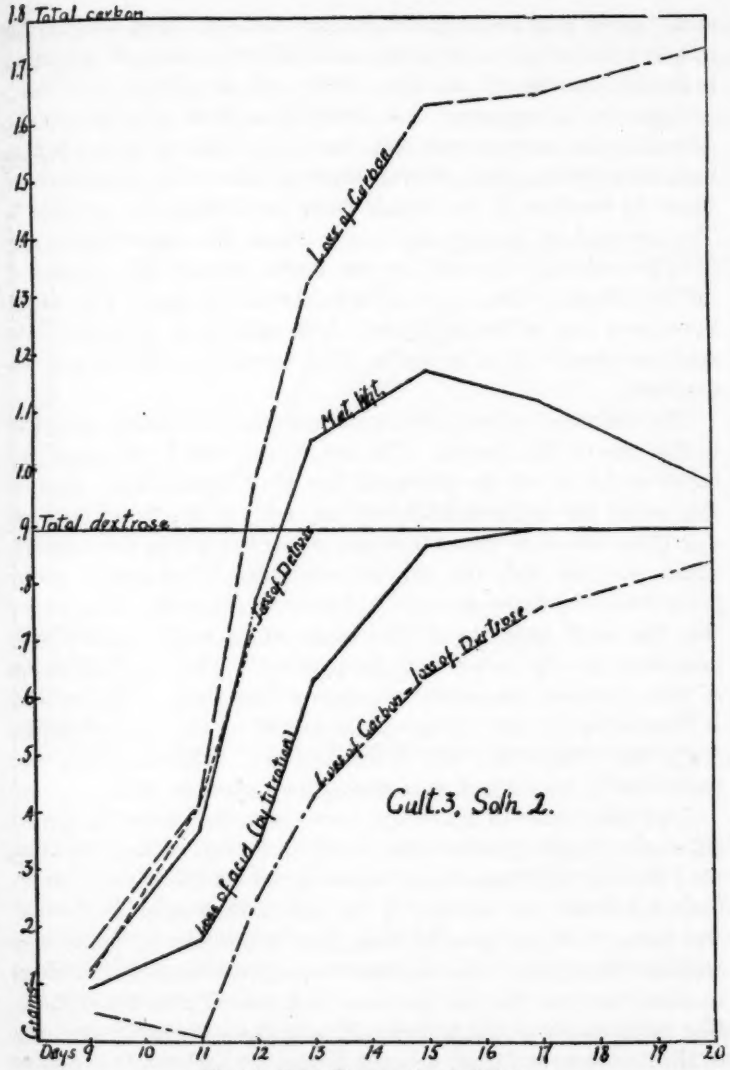


Fig. 3. *Penicillium* sp. in solution 2.

1, a point was reached at which the loss in weight due to autolysis just about balanced the increase due to the utilization of the

small amount of dextrose left in the solution. Mat weights for solution 2 showed a normal rise and fall in this respect, attaining a decided maximum and then falling off steadily.

Sugar had disappeared completely from both solutions on the fifteenth day after inoculation, but if the time is considered as beginning when good growth started, then the utilization of sugar in solution 2 was much more rapid than in solution 1. The amount of mat at the point when the sugar completely disappeared from the solution was much greater in solution 2 (about 1.5 gm.) than in solution 1 (about 0.62 gm.). This might have been due to the additional citric acid used or to the citric acid combined with a favorable effect upon the utilization of the dextrose.

The utilization of the citrate radical was remarkably complete in the case of this fungus. The amount of acid by titration had fallen to 2.1 cc. on the sixteenth day after inoculation. Beyond this point the utilization slowed up, but on the twenty-second day there was only about 0.05 gm. of carbon left in the solution, which was less than the amount remaining in solution 1 at the point when the dextrose could no longer be detected. This means that the small quantity of the citrate which was combined with potassium as the cation had disappeared. That the utilization of this combined compound was slower than that of the free acid is illustrated by the falling off in weight of the mat while the combined citrate was still being utilized. This fungus would undoubtedly be classed as a strong user of citric acid.

Aspergillus sp.—In figs. 4 and 5 are found the curves for *Aspergillus* sp. Rapid growth in the 2 solutions began about the same time and the determinations were started on the second day in both solutions. In solution 2 the maximum weight of the mat was about 0.35 gm. greater than that in solution 1, an increase of about two-fifths. This increased maximum weight was attained at about the time that the dextrose disappeared from the solution. The additional carbohydrate would seem to serve as an auxiliary to the dextrose, but in its absence it was not sufficiently available to keep up the increase of weight, since the weight commenced to decrease while the citric acid was still being used. The dextrose, however, was more rapidly used in solution 2 than in solution 1.

This may have been due to the fact that the solution was buffered at a P_H favorable for the utilization of the dextrose, and that this buffering stabilized the solution against the effect of waste products on the reaction. This is well borne out by a comparison of the data on the P_H of the solutions, since on the fourth day solution 1 had reached a reaction of P_H 3.8, and solution 2 a re-

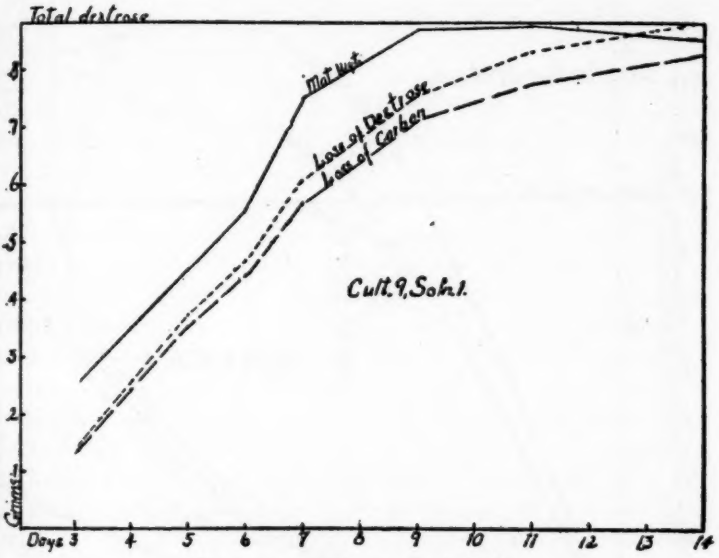


Fig. 4. *Aspergillus* sp. in soiution 1.

action of 2.6, and while from the fourth to the tenth day the reaction of solution 1 only reached P_H 3.9, solution 2 on the tenth day had a P_H of only 3.0, the progress in an alkaline direction having been very gradual. The difference was not very great but might be sufficient to account for the more rapid utilization of dextrose in solution 2.

The utilization of carbon in solution 2 did not indicate a very rapid utilization of citric acid, nor did the titration figures indicate it. Had the experiment been run longer a much larger amount of citric acid would probably have been used. The titration for the blank was 46.6 cc. of N/10 NaOH (for 25 cc. of the diluted

solution), and the titration at the time of the last determination was 26.1 cc. N/10 NaOH on the same amount of solution, indicating that less than half of the acid had been used. The difference

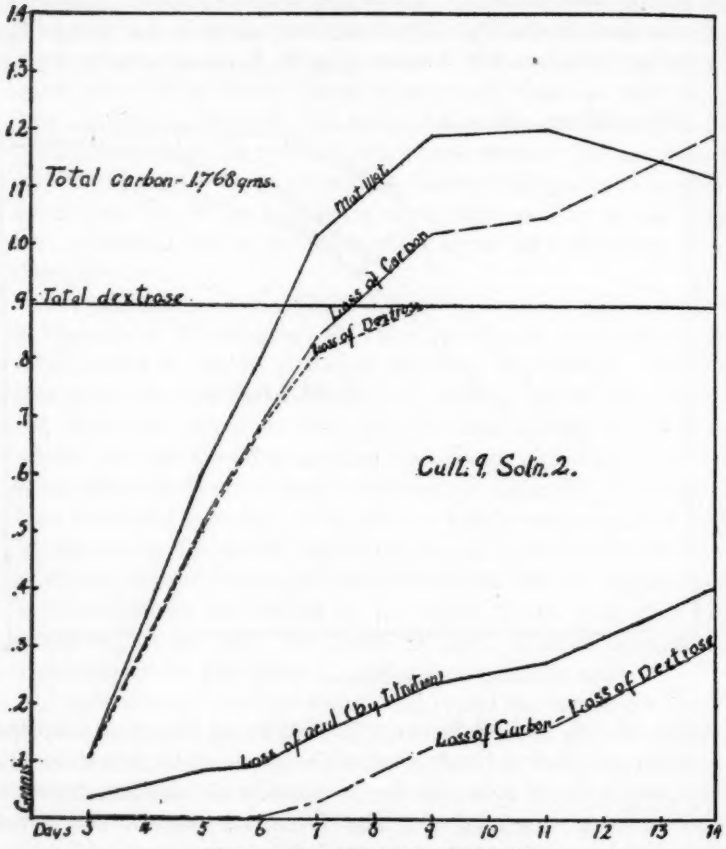


Fig. 5. *Aspergillus* sp. in solution 2.

between the total carbon-dextrose curve for cultures 9 and 3 is very marked, the rise of this curve being very rapid in the case of organism 3 and very slow in the case of organism 9.

Penicillium stoloniferum.—In figs. 6 and 7 are found the curves for the metabolism of this fungus, and the curves for the trend

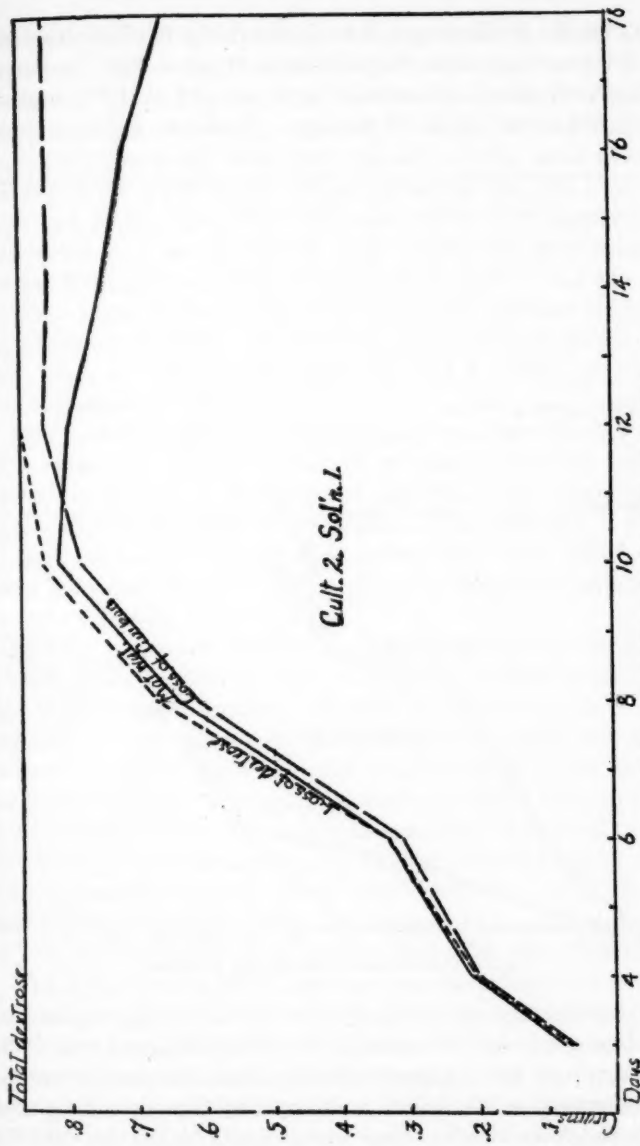


Fig. 6. *Penicillium stoloniferum* in solution 1.

of P_H for the 2 solutions will be found in fig. 8. For this fungus the solution was made slightly more alkaline than in the preceding work, solution 1 containing 20 cc. of 2 *N* H_3PO_4 and solution 2, 175 cc. of 2 *N* KOH per liter. So closely did the duplicate

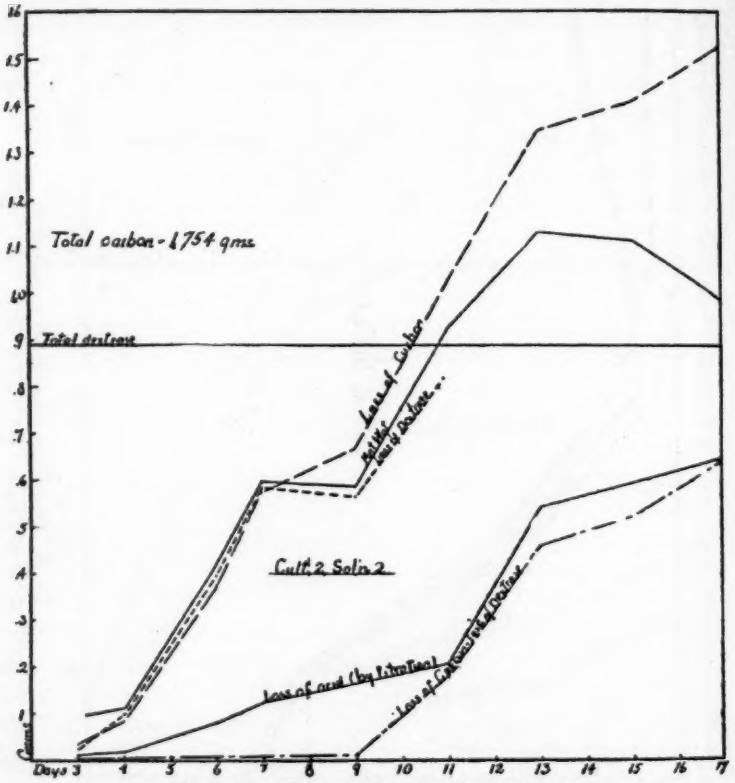


Fig. 7. *Penicillium stoloniferum* in solution 2.

mats and chemical analyses agree in the case of organisms 3 and 9, and so great was the amount of routine required that it was decided to mix the duplicate solutions in each case and run one determination on the mixed solution in all the succeeding work.

The mat from solution 2 was about two-fifths heavier than that from solution 1, as was also the case with organism 9, and the

maximum weight occurred very shortly after the dextrose disappeared. Following the maximum there was a steady decline in solution 1, but an even more rapid decline in solution 2, which is not so easily explained, especially as the indications are that citric acid was being used fairly rapidly at the same time. In the curves for solution 2 is noted a break at the fifth to seventh day, and at the same time the mats which had become rather gray-green with spores showed signs of renewed growth and the formation of tufts of white mycelium took place. This was noted at other times under similar conditions, but whether it had any significance is doubtful. However, it was peculiar that this should take place at a time when there was also a marked increase in the utilization of citric acid.

The final disappearance of dextrose from the solution was a little slower in solution 2 than in solution 1, but the mat was heavier in solution 2 at this point than that in solution 1 at the same point in the course of metabolism. This indicated a sparing of dextrose due to the presence of the citric acid, which might have been due to the buffering at a P_H somewhat unfavorable to the utilization of dextrose.

The curves for the trend of P_H (fig. 8) showed that in solution 1 there was a marked increase in alkalinity as soon as the fungus had begun to grow rapidly. In solution 2, however, as would be expected in such a strongly buffered solution, there was a long-maintained curve at nearly the original acidity, the trend of alkalinity coming very rapidly toward the end of the experiment and coordinate with it a rapid decrease in the weight of the mat. According to data obtained later, if solution 2 had been left longer a P_H approaching closely to 9.0 would have been attained. This final falling off should have been coincident with the disappearance of free citric acid and most of the acid salts from the solution.

The utilization of citric acid here was very complete and compared favorably with the utilization by *Penicillium* sp. (fig. 3). There was considerable loss of acid before the dextrose had disappeared from the solution, and although there continued to be a loss of acid after the disappearance of dextrose the mat steadily declined in weight, indicating that citric acid by itself was probably not very efficient as a source of carbon.

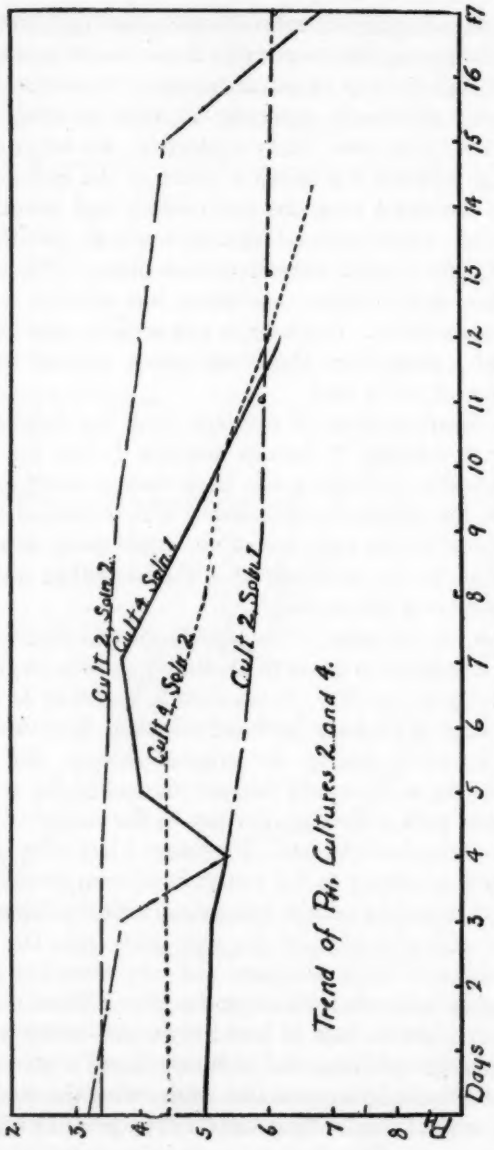


Fig. 8. Trend of PH for *Penicillium stoloniferum* and *Diplodia natalensis*.

The curve for the "loss of carbon—loss of dextrose" remained very constant around zero until the ninth day when it started to rise very rapidly, indicating that citric acid was being utilized; as will be seen, this corresponded with the figures for the titration of the free acid.

Penicillium digitatum.—The curves for the weights of the mats are given in fig. 8, the analytical data in figs. 9 and 10. Owing to the fact that *P. digitatum* grows very little with inorganic nitrogen sources, peptone was used instead of KNO_3, an equivalent amount of the peptone solution formerly made up (see p. 245) being used. According to the figures on the carbon determinations, peptone gave about 25 per cent oxidizable carbon under the conditions of the determinations. The amount of peptone added was so small, however, as compared to the amount of carbon present that no attempt was made to remove this substance from the solution in making analyses. In the case of sugar determinations it is probable that there are small amounts of copper-reducing substances in peptone but the error is so small, and the removal of peptone before the determinations without taking out some of the sugar is so difficult, that the determinations were run without removing the peptone from the solution.

In solution 2 separate analyses were made of the citric acid in the following manner: The acid was precipitated by the barium method as previously described and the precipitate dried. The dry precipitate was dissolved in warm concentrated H_3PO_4 and transferred to the reaction flask of the carbon-determination apparatus and the carbon determined in the usual way. As soon as the H_2SO_4 was added $BaSO_4$ was thrown down, but this caused no difficulty in carrying out the determinations. The peptone gave a little interference in this procedure but this did not amount to more than 3–6 mgms. of carbon per determination of 75–100 mgms. of carbon. This was done because citric acid had been found to disappear in the preliminary cultures and little growth had resulted.

The growth curves shown in fig. 9 show that as far as the 2 solutions were concerned there was little difference in value, if the weight of mat be used as the criterion. Solution 2 produced about the same growth as solution 1, but was a little slower in

doing so and the final decline in weight was correspondingly slower. The dextrose had disappeared 2 days earlier in solution 1 than in solution 2, and if the mat curves were smoothed out there would be about that much difference between the times of attaining the maximum weight of mat in the 2 solutions. Apparently these maximum weights had been attained shortly before the dextrose was completely used up. This is uncertain, however,

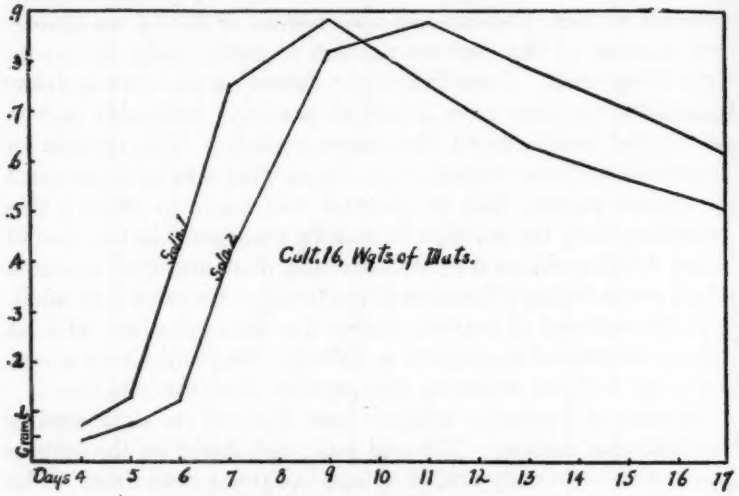


Fig. 9. *Penicillium digitatum*, weight of mats.

since in such a case as that in fig. 10 there was a slight amount of dextrose on the eighth day and none on the tenth day, but if the curve for the loss of dextrose be calculated according to the conformation of the curve previous to the eighth day, the disappearance of dextrose would be found to occur some time on the eighth day or near the beginning of the ninth day.

The presence of the peptone in the solution complicates the problem of drawing conclusions from the data obtained. The remarkable fact in connection with solution 1 (fig. 10) is that the curve for the loss of carbon seems to tally almost absolutely with that for the loss of dextrose, and would consequently make it appear that little or none of the easily oxidizable carbon in the

peptone was utilized. This was probably not the case, however, and this is further indicated by the difference between the curve for "loss of carbon—loss of dextrose" and the loss of citric acid as determined by analysis (fig. 11). The P_H of solution 1 ran from 5.5 at the beginning to 4.1 and back to 6.4 where it remained for most of the course of the experiment.

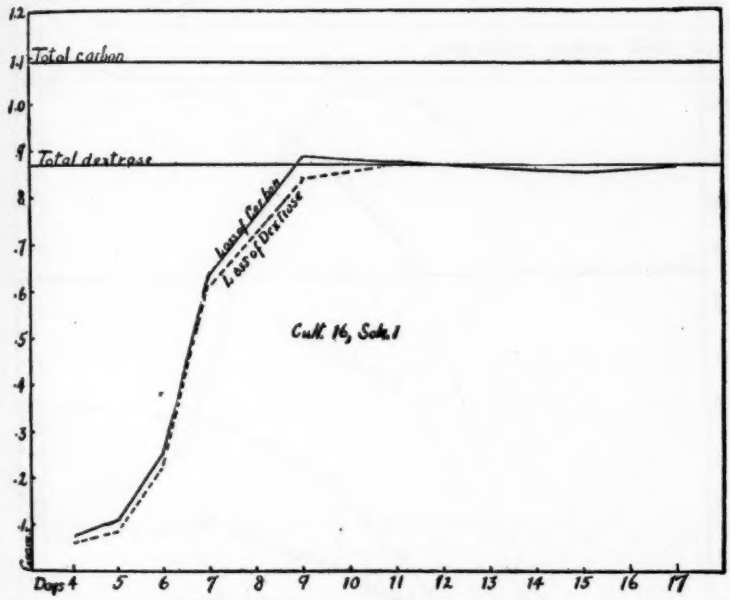


Fig. 10. *Penicillium digitatum* in solution 1.

It is impossible to draw clean-cut conclusions from the curves in fig. 11. The fact that the loss of citric acid as determined by analysis was greater than the "loss of carbon—loss of dextrose" would indicate that some product was being formed in the solution from either the citric acid or the dextrose, and, in all probability, from the former. That this substance could not be oxalic acid is obvious from the fact that by the method used oxalic acid would have been included with the citric acid in the barium precipitation. It was not a volatile substance, since it was oxidized in the carbon apparatus and, if an acid at all, it had a

soluble barium salt and very weak acidity, for the titration figures fell to 1.1 cc. of N/10 NaOH per 25 cc. of solution, and the P_H to 7.2. These facts preclude most of the common acids and give the impression that it was some other inert substance.

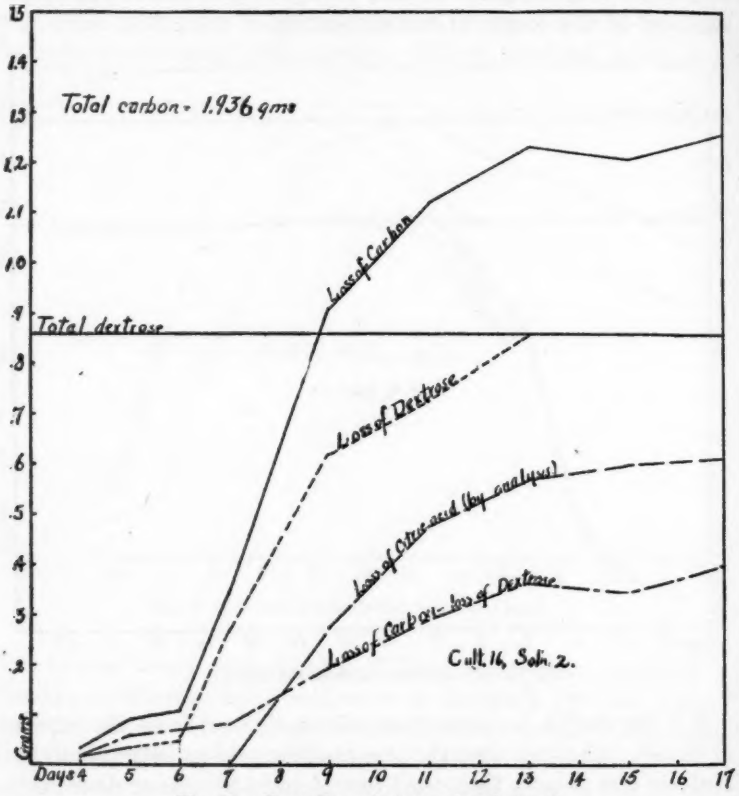


Fig. 11. *Penicillium digitatum* in solution 2.

Diplodia natalensis.—In figs. 12 and 13 are found the curves for the analytical work and in fig. 8 the curves indicating the trend of P_H during the period of growth. The solution used for this fungus was the same as that used for organism 2 except that NH_4NO_3 was substituted for KNO_3.

There was considerably greater growth in solution 2 than in

solution 1, almost one-half more, but it is noticeable that the peak of growth occurred after all the dextrose had disappeared, in solution 2, but was coincident with its disappearance in solution 1, or approximately so. Likewise, the weight of the mats fell off much more rapidly in solution 1 than in solution 2. Here again there was a greater weight of mat in solution 2 when the dextrose disappeared than in solution 1 at the same relative period.

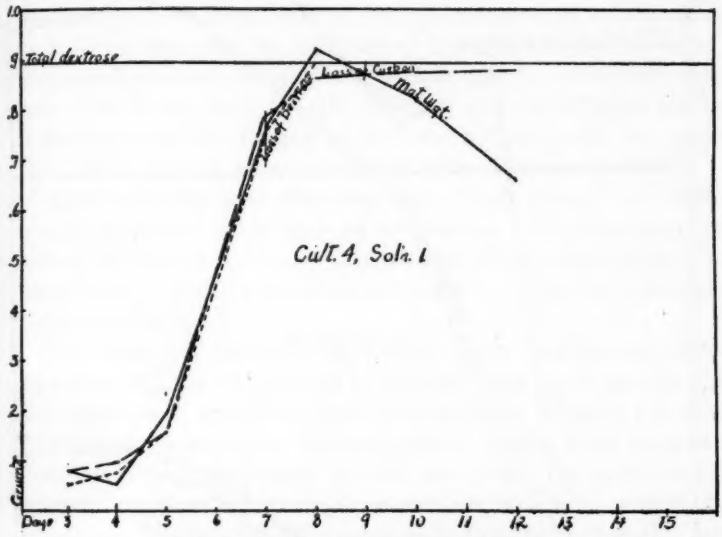


Fig. 12. *Diplodia natalensis* in solution 1.

The disappearance of dextrose came at about the same relative period, in both solutions. The data for solution 1 would indicate that little, if any, non-volatile compounds were formed. The "loss of carbon—loss of dextrose" curve for solution 2 proceeds below the base line for the first 8 days, indicating that a small amount of waste products was probably formed from the dextrose. Beginning on the ninth day this curve begins to rise rapidly, and from this point runs practically parallel to the curve calculated from the titration figures. Likewise, the point at which the curve for "loss of carbon—loss of dextrose" starts to ascend is coincident with the final disappearance of dextrose from the solution.

It was impossible to detect even traces of oxalic acid in this solution or of either citric or oxalic in the dextrose solution. However, in neutral solution, a gelatinous precipitate could be obtained

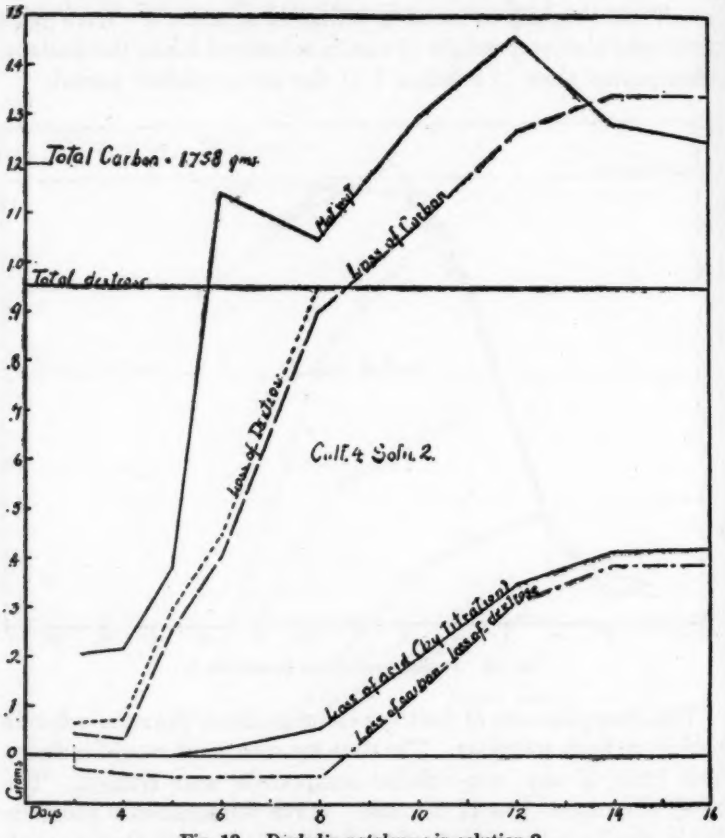


Fig. 13. *Diplodia natalensis* in solution 2.

with $Ca(OOC.CH_3)_2$. This precipitate had a wine-red color and dissolved with difficulty in dilute acids, did not reduce Fehling's, give a pentose reaction or show either alkaline or acid characteristics. Moreover, there seemed to be a considerable amount of gelatinous material in the solution in which the fungus was grow-

ing, making it very difficult to filter off the mat, the supernatant liquid going very slowly through coarse filter paper.

The curves for the trend of P_H are found in fig. 8. Solution 1 showed a marked rise of acidity followed by a falling off to alkalinity. Solution 2 did not rise in acidity but after 6 days began to fall off steadily toward alkalinity. Whether the development of acid was hindered by the buffer action of solution 2 or merely masked by it is a question that cannot be answered satisfactorily. It is to be seen that as the titer of the solution decreased to a very small amount, utilization of carbon ceased. This might have been due to the fact that the solution was too alkaline for the metabolism of this fungus or that the fungus could not utilize the citrate radical when combined with potassium.

Alternaria Citri and *Alternaria* sp.—These 2 fungi will be discussed together for the sake of comparison. The solutions contained KNO_3 as a source of nitrogen and 100-cc. flasks were used. In diluting, 2 flasks were made up to 200 cc. The P_H of the solution was about 4.7.

It is difficult to interpret the weight curves for these organisms, as seen in fig. 14. Organism 14 showed more rapid growth than did organism 7, and if the final high figure for solution 1 is taken into account would seem to have made a greater total growth on dextrose alone. However, as will be noted, the dextrose had entirely disappeared 16 days before this final high weight was obtained. There are only 2 reasonable explanations of this fact: the first, that this weight is accounted for by the irregularity of growth, and that these 2 particular mats would have weighed as high or higher at an earlier period; the second, that the mats for some reason were insufficiently dried and consequently were somewhat heavier than they should have been. While this last-mentioned explanation seems improbable, since the mats were run in large groups and no other difficulties were encountered, yet it seems wise to disregard such an unusually high weight occurring at this point. With regard to both of these fungi and other fungi of a similar nature there is considerable irregularity in the weights of the mats, presumably due to the slowness of growth, and consequently the greater likelihood of being influenced by factors of the environment. It would have been highly

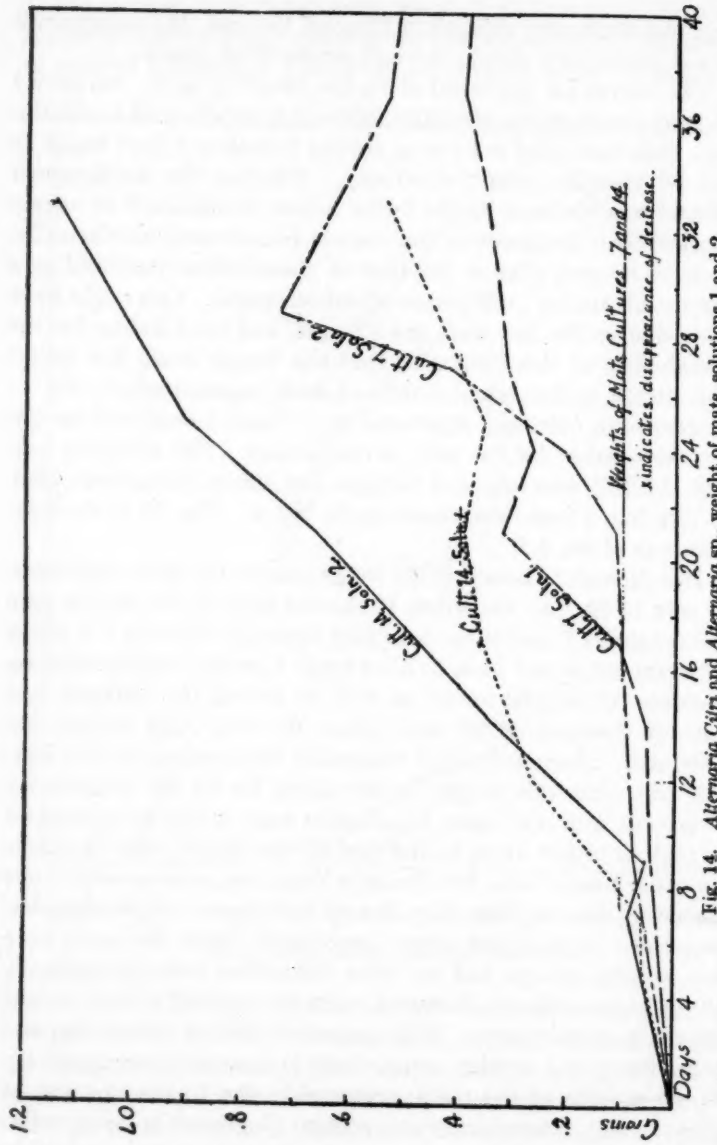


Fig. 14. *Alternaria Citri* and *Alternaria* sp., weight of mats, solutions 1 and 2.

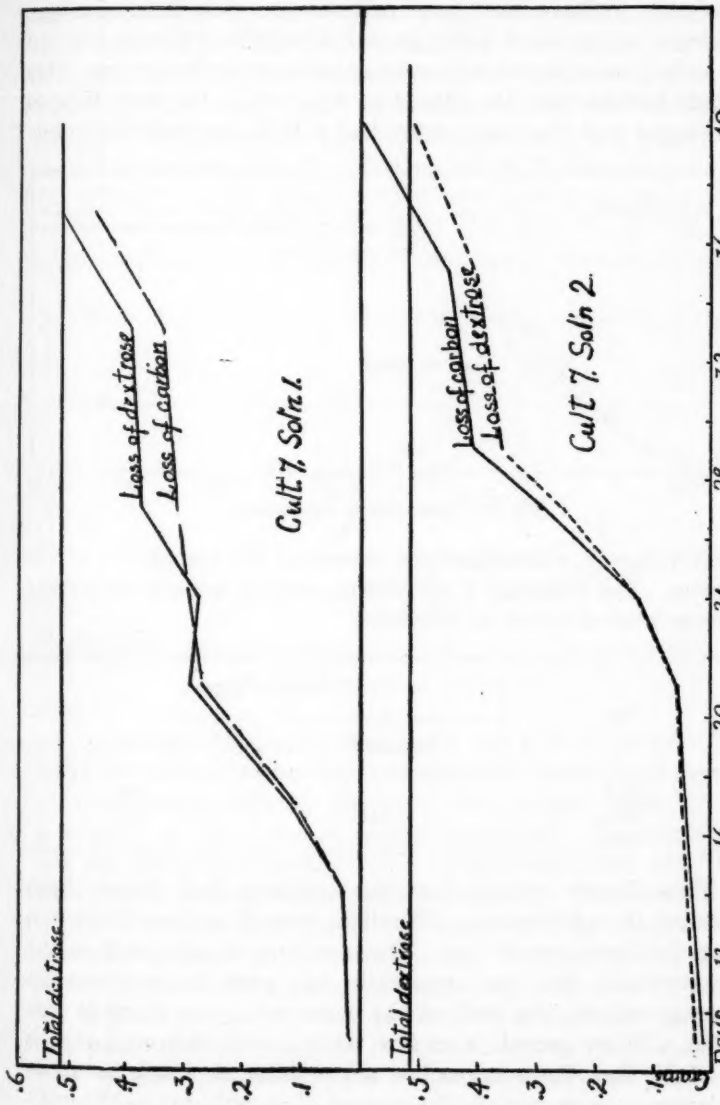


Fig. 15. *Alternaria Citri* in solution 1 (above) and solution 2 (below).

desirable to have had more cultures for each determination. Culture 14 gave much better growth in solution 2 than in solution 1, as well as a delayed utilization of dextrose in the former. This would indicate that the citric acid was used at the same time as the sugar and that the former had a high mat-building value.

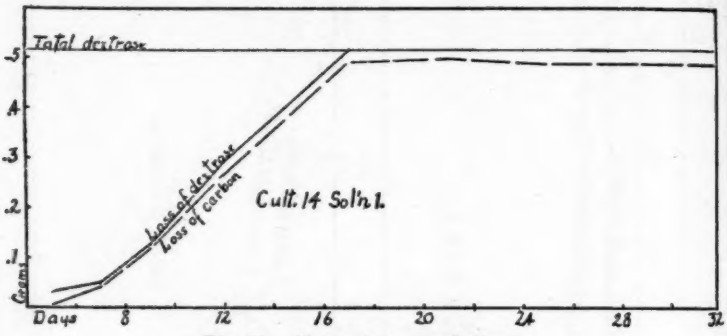


Fig. 16. *Alternaria* sp. in solution 1.

Unfortunately, contaminations prevented the completion of the curves. For organism 7 additional weights not shown on the curves were obtained as follows:

Day	Wgts. in mgms.	
	Solution 1	Solution 2
43rd		502
47th	341	486
50th	376	

These figures indicate that the maximum had already been reached, though there was still a slight trace of dextrose in solution 2 on the forty-seventh day. The maximum in solution 2 on the twenty-ninth day was apparently too great to represent an average culture, the peak of the curve being too sharp at this point. Where growth is as slow as it is with this organism, it is likely that there is reached a condition in which autolysis balances or even exceeds the amount of growth due to the utilization of the carbohydrate still remaining in the solution. It

seems reasonable to suppose that the smaller the amount of carbohydrate in the solution, in proportion to the absorbing surface, the more difficult it becomes for the fungus to absorb it in sufficient quantities to show a continued increase in growth. Where growth is very rapid, as in some species of *Penicillium*, this effect would be masked by the rapid and complete disappearance of the sugar from the solution, but in the case of *A. Citri* this difficulty would manifest itself to a greater degree.

The curves representing the daily change in P_H of the two solutions of organism 14 (figs. 16, 17) are very interesting and prob-

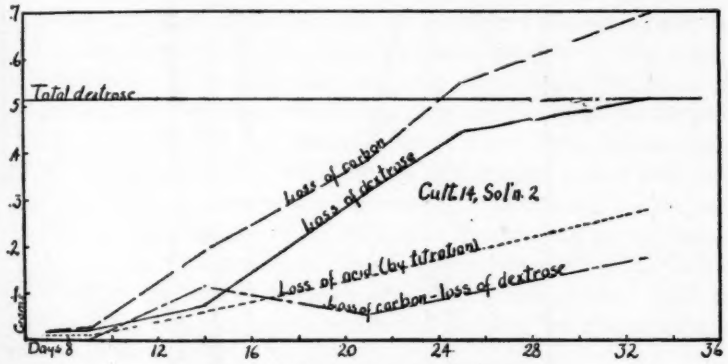


Fig. 17. *Alternaria* sp. in solution 2.

ably represent the typical results for this type of fungus. The curve for solution 1 rises very slightly and then falls off rapidly to a comparatively high alkalinity and remains there for the remainder of the duration of the experiment. In solution 2 the P_H remained constant for a considerable time and then showed a very slow falling off toward alkalinity. This maintaining of the P_H in the early stages of metabolism is an expression of the buffer activity of the citrate mixture; the rapidity with which the subsequent falling off occurs depends entirely upon the rapidity with which the citric acid is used. If in this case the buffer was not consumed at all by the fungus the stability would be even greater and an organism would be required to produce a considerable amount of either alkaline or acid substances to move

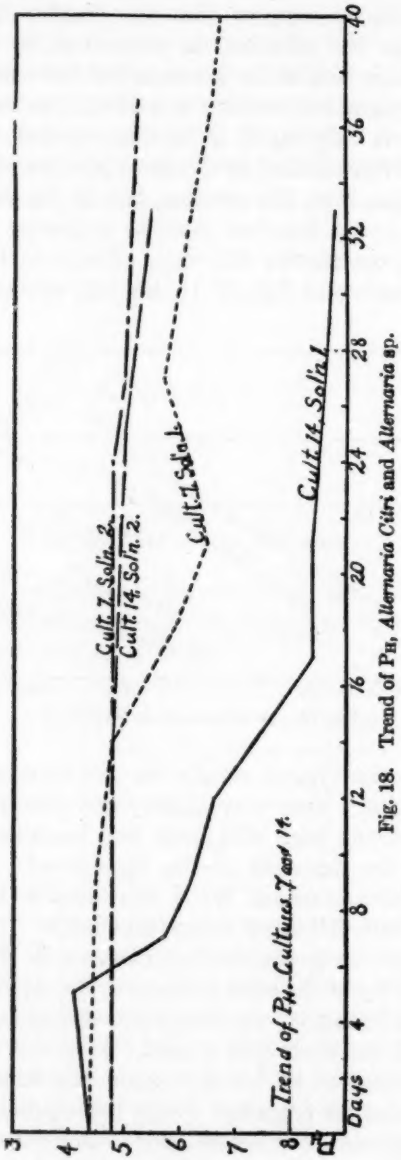


Fig. 18. Trend of PH, *Alternaria Citri* and *Alternaria* sp.

the P_H through any considerable range of hydrogen-ion concentration. In solution 2, culture 7, we find the same buffering, but there is a marked difference between the curve for solution 1 and that for organism 14, solution 1. There was no rise of acidity at the start but a gradual falling off followed by a rise to a slight peak and a subsequent falling off. The rise in acidity at this late point might be considered only a variation in certain particular cultures were it not for the fact that several determinations were involved in this "peak."

For organism 14, solution 2, there was a steady rise in the "loss of carbon—loss of dextrose" curve, almost from the start. This rise is very slow, however, and the increased growth in solution 2 seems to be out of proportion to the amount of carbon used to produce it. How much the buffering rather than the use of the carbon from the citrate may account for this increase is a question that cannot safely be discussed until more data are available.

Generally speaking, organism 14 is the more active and rapid grower when compared with organism 7; likewise it makes more effective use of the citrate radical. Another marked difference between the two organisms lies in the curve of P_H in a medium in which dextrose is supplied alone, as noted above. Whether these fungi represent two species is a problem for the mycologist to decide however.

Sclerotinia Libertiana.—The growth curves for organism 11 are found in fig. 19 and the curves for the analyses in figs. 20–21. Owing to the slow growth of this fungus and the succeeding one (*Phomopsis Citri*) the culture solution was varied somewhat. Peptone was used as a source of carbon and solution 2 contained M/20 dextrose instead of M/4. Flasks of 100 cc. capacity were used instead of the 300-cc. flasks.

In the growth curve for solution 1 a sharp peak appears on the ninth day and coincident with it is found a peak in the "loss of carbon" curve and a complete loss of dextrose. On the ninth day it was assumed that there was no dextrose in the solution but the curve for "loss of carbon" indicates that there was probably considerable sugar present and that the peak on the ninth day indicated erratic cultures. In the normal culture the dextrose would probably be found to disappear about the fourteenth to

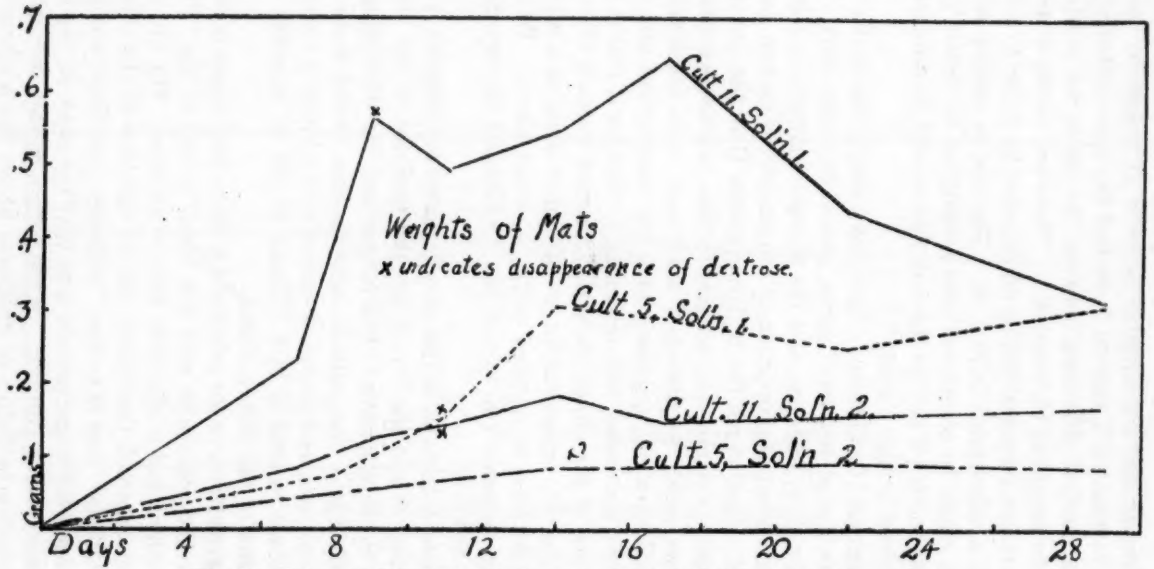


Fig. 19. *Sclerotinia Libertiana* and *Phomopsis Citri*, weight of mats, solutions 1 and 2.

sixteenth day under the same conditions. If no citrate were used the growth curve for solution 2, at its peak, would be expected to be about one-fifth the height of that for solution 1. As a matter of fact it is a little better than that and the peak is maintained instead of falling off as it did in solution 1. The curves

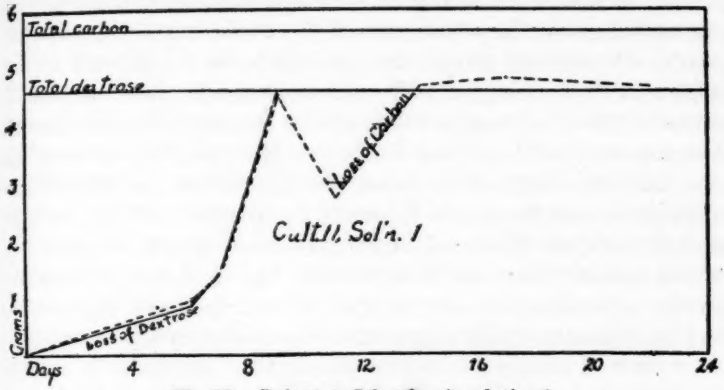


Fig. 20. *Sclrotinia Libertiana* in solution 1.

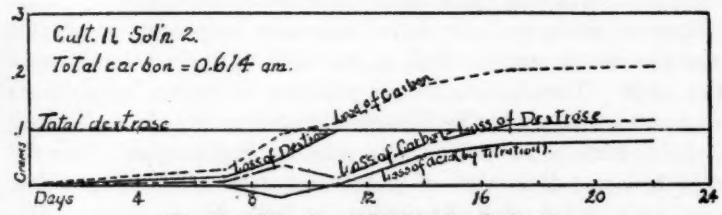


Fig. 21. *Sclerotinia Libertiana* in solution 2.

for the analyses indicate that a little citrate was used though not a great deal.

In the analytical curves for solution 1 (fig. 20) the "loss of dextrose" and "loss of carbon" curves follow each other closely, and in solution 2 the "loss of carbon—loss of dextrose" curve rises more rapidly than does the curve calculated from the titrations. The conclusion from the above circumstances is that the salt (*in toto*), as well as the free acid, was being used.

Phomopsis Citri.—The growth curves for organism 5 are given in fig. 19. The analytical curves are not given, as they held noth-

ing of particular interest, since, at the end of 22 days, no more than a trace of citrate had been used. The maximum weight of mat for solution 2 was a little more than a fifth of the maximum in solution 1. On the fourteenth day a good test for oxalic acid was obtained in solution 1.

The production of alcoholic products by various fungi.—In connection with the latter part of the work it was intended to carry out aeration experiments in which the fungi were to be grown in flasks plugged with rubber stoppers provided with 2 aeration tubes (inlet and outlet) and by this method to determine the amount of CO_2 produced. It was believed that by aerating the cultures twice each 24 hours the CO_2 tension would be kept sufficiently low to allow of normal development of the fungus and that enough O_2 would be furnished. However, organism 9, which was tried first, failed to develop rapidly and for a time refused to sporulate, and later sporulated only sparingly in contrast to the ordinary cotton-plugged cultures which sporulated heavily. As it seemed useless to continue the aeration experiment, due to the abnormal growth resulting from these conditions, the flasks were left stoppered for about a week without aeration. As the culture solution had developed a strong fermentative odor, it was poured off and distilled, in the hope of finding the cause of the odor. Twenty-five cc. of solution of markedly alcoholic odor were obtained by distillation from 150 cc. of culture solution; and on dilution to 100 cc. the solution had a specific gravity slightly lower than that of water and gave a benzoate with a very penetrating odor (Baumann and Schotten reaction). This odor somewhat resembled that of ethyl benzoate but seemed more penetrating. Weak iodoform tests were obtained in the cold and stronger on warming, following the instructions of Mulliken ('04). The crystals were examined microscopically and found to correspond with those given by ethyl alcohol by the same test but this is not distinctive. No similar products were obtained where only dextrose was present as the source of carbon. Lack of time and equipment for organic analysis prevented further study of this solution in an analytical way, but similar phenomena were observed in numerous cultures and it may be worth while to indicate some of the results obtained.

In cultures of *Phomopsis* in the presence of large amounts of citrates a marked yeasty smell suggesting ethyl alcohol and dilute acetic acid developed after a prolonged growth period; with only dextrose and peptone no such odor developed. Both of the *Alternaria* spp. in dextrose-citrate mixtures developed similar odors, and distillation gave solutions with the odor of alcohol. These distillates gave iodoform tests on warming and benzoates with the odor of ethyl benzoate, or suggesting mixtures of ethyl benzoate with allied benzoates. When a series in which KNO_3 was used as the source of nitrogen was inoculated with *Oospora Citri-aurantii* the growth was so slow and the difficulties connected with filtering off the organism so great that the series had to be abandoned. After about 2 weeks a very marked sweetish odor developed, nearly resembling slightly fermented cider, and this applied in less degree to the culture solution where only dextrose was used as the source of carbon. On distillation the odorous compound was carried over to the distillate and suggested an ester of ethyl or some closely allied alcohol.

These results are necessarily incomplete, yet merit some discussion. The factors which all these cases had in common were (1) a growing condition in which there was a tendency to anaerobic environment, and (2) citric acid-potassium citrate mixtures (with one exception—see *Oospora Citri-aurantii*). This semi-anaerobic condition was obtained in the *Aspergillus* cultures through stoppering the flasks with rubber stoppers and in the other cultures by the mat tending to form (where there was a mat formed) *in* the solution instead of *on* its surface as was usual with most of the fungi used. *Oospora* grows in the solution in a yeast-like manner; the two species of *Alternaria* and the *Phomopsis*, besides being slow growers, start under the surface of the solution and eventually form a sponge-like mass which contains the solution interstitially. Under such circumstances there is certain to be a solution saturated with CO_2 and at the same time a probable shortage of O_2 due to the slow diffusion from the air into the solution. A moderated condition of anaerobism in which there is an excess of CO_2 and a shortage of O_2 would seem then to be a primary factor.

The question of carbon source is more complicated than is

indicated at first glance. There are 2 solutions under consideration; one in which only dextrose was used as a source of carbohydrate, the other with dextrose and the addition of a comparatively large amount of citric acid-potassium citrate mixture. The alcoholic products occurred only where the citrate mixture was present, except in the case of *Oospora*, and the natural inference is that the citrate radical is the source of the products formed. However, it is within the field of possibility that this may not be the case, but that the strong buffer action of the citrate mixture is successful in maintaining the reaction at an unfavorable P_H for a considerable time, which might result in an abnormal metabolism of dextrose. However, in the *Phomopsis* cultures only a small amount of dextrose was present with the citrate mixture and this was soon utilized, probably before the solution had acquired the semi-anaerobic condition. Likewise, when the cultures of *Aspergillus*, which had been stoppered for the aeration work and which contained only dextrose and produced no appreciable amount of alcoholic products, had citric acid added to them after the dextrose was exhausted the odor of alcohol rapidly developed.

The question of buffering was raised primarily in connection with *Diplodia natalensis*. A series of flasks of the dextrose-citrate solution was prepared but made too acid for the good growth of this fungus. After inoculation the fungus developed very slowly tufts of floating mycelium in the solution and after about 2 weeks a small tuft on the surface. The odor of ethyl alcohol was very ttrong, and there was also present some other substance of penelrating odor but probably not acetic acid; on distillation the distilsate gave an immediate and very strong iodoform test in the cold, indicating that either acetone or isopropyl alcohol was probably present. As no benzoyl chloride was available at the time no benzoate was made. The odor of the distillate, however, suggested a mixture of ethyl alcohol and acetone together with some other substance. A similar solution adjusted to a more favorable P_H did not produce these odorous compounds. The inference here is that the buffering of the solution at an unfavorable P_H might be the important factor, but it is reasonable to suppose that the buffering should affect the metabolism of the citrate

radical as well as of the dextrose. Moreover, the results might be indirect in that the buffering at an unfavorable P_H caused the mat to form *in* instead of *on* the solution and that this was the direct cause of the incomplete metabolism.

Other cultures used failed to produce such evident volatile metabolic products, but both *Penicillium stoloniferum* and *P.* sp. produced some odorous compound which was given off when the solution was neutralized. This distilled over readily and formed a benzoate by the Baumann and Schotten reaction which had an odor of some rubber compounds.

These observations are very suggestive of a fruitful field for investigation in the future. Moreover, they merely add to the data indicating that the metabolism of fungi is far from a simple problem and that the end products may be in many ways independent of the conditions of the environment. It is quite probable that ethyl and isopropyl alcohols, acetic acid, and possibly acetone may be end products of the metabolism of citric acid by fungi and that they may also be the end products of the metabolism of sugars, providing the correct environmental conditions are provided. It is likewise probable that these environmental conditions have to do with the O_2–CO_2 relations and that any factors affecting this relation may have effect upon the ultimate products obtained.

Summary

An improved method for the determination of citric acid, especially applicable to culture solutions, has been offered.

The application of the wet combustion method, for the determination of total carbon, to physiological work has been indicated.

A number of fungi which attack citrus fruits have been studied with regard to their ability to utilize citric acid as a source of carbon with the following general results:

(1) None of the fungi tried was found to thrive on citrate as the sole source of carbon.

(2) Citrate mixtures adjusted to a favorable P_H proved to be efficient supplementary carbon sources, when used with small quantities of dextrose, for all the fungi used with the exception of *Penicillium digitatum* and *Phomopsis Citri*.

(3) After a mat had been grown on dextrose, free citric acid was utilized readily by *Penicillium stoloniferum*, *P.* sp., and *Aspergillus* sp., and somewhat less readily by *Sclerotinia Libertiana*. Free citric acid was used slightly or not at all by mats of *Diplodia natalensis*, *Phomopsis Citri*, *Alternaria Citri*, *Alternaria* sp. and *Penicillium digitatum*. The response of the fungi in this respect was coordinate with their tolerance of the hydrogen-ion concentration.

Studies have been carried out with *Penicillium* sp., *Penicillium stoloniferum*, *Penicillium digitatum*, *Aspergillus* sp., *Diplodia natalensis*, *Alternaria Citri*, *Alternaria* sp., *Phomopsis Citri*, and *Sclerotinia Libertiana* on a dextrose and a dextrose-citrate medium, and curves for the growth, trend of P_H, "loss of dextrose," "loss of carbon," etc., are given for these fungi.

Acidic and alcoholic products were found to be formed under unfavorable environmental conditions, that is, lack of O_2, unfavorable P_H, etc.

It has been pointed out that tolerance or utilization of free citric acid is probably not an important factor in the specialized parasitism of such fungi as *Phomopsis Citri*, *Penicillium digitatum*, *Alternaria Citri*, *Alternaria* sp., *Sclerotinia Libertiana*, and *Diplodia natalensis*, but that it probably is a factor in the destructive rotting of injured fruit by numerous fungi which cause this final collapse, and that under suitable environmental conditions *Penicillium stoloniferum*, *Penicillium* sp., and *Aspergillus* sp. would probably come in the latter category.

It is with great pleasure that the writer takes this opportunity for thanking Dr. B. M. Duggar for invaluable aid and advice in the prosecution of the work here reported; he also wishes to thank Dr. P. A. Shaffer of the Medical School of Washington University for aid in connection with the chemical work, and Dr. George T. Moore for the privileges and facilities of the Missouri Botanical Garden.

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Bibliography

Amberg, S., and McClure, W. B. ('17). The occurrence of citric acid in urine. Am. Jour. Physiol. **44**: 453–462. 1917.

Assoc. Offic. Agr. Chemists ('07). Official and provisional methods of analysis. U. S. Dept. Agr. Bur. Chem. Bul. 107: I-XXII, 1-230. *f. 1-11.* 1907. (See p. 81).

————. ('19). Official and tentative methods of analysis. I-XII, 1-417. 1919. (See p. 159).

Ayers, S. H., and Rupp, P. ('18). Simultaneous acid and alkaline bacterial fermentations from dextrose and the salts of organic acids respectively. Jour. Infect. Dis. **23**: 188-216. 1918.

Bacon, R. F., and Dunbar, P. B. ('11). Changes taking place during the spoilage of tomatoes, with methods for detecting spoilage in tomato products. U. S. Dept. Agr., Bur. Chem. Circ. 78: 1-15. 1911.

Bartholomew, E. T. ('23). Internal decline of lemons. II. Growth rate, water content, and acidity of lemons at different stages of maturity. Am. Jour. Bot. **10**: 117-126. 1923.

Baumann, E. ('86). Ueber ein einfache Methode der Darstellung von Benzoesaureäthern. Ber. d. deut. chem. Ges. **19**: 3218-3222. 1886.

Bear, F. E., and Salter, R. M. ('16). Methods in soil analysis. W. Va. Agr. Exp. Sta., Tech. Bul. 159: 1-24. 1916.

Bigelow, W. D., and Dunbar, P. B. ('17). The acid content of fruits. Jour. Ind. and Eng. Chem. **9**: 762-767. 1917.

Blasdale, W. C. ('18). Principles of quantitative analysis. 402 pp. *70 f.* 1918.

Burger, O. F. ('20). Decay in citrus fruits during transportation. Calif. Dept. Agr., Monthly Bul. **9**: 365-370. 1920.

Butkewitsch, W. ('22). Über die Bildung der Citronen- und Oxalsäure in den Citromyces-Kulturen auf Zucker und das Verfahren zur quantitativen Bestimmung dieser Säuren. Biochem. Zeitschr. **131**: 327-337. 1922.

————. ('22a). Über den Verbrauch und die Bildung der Citronensäure in den Kulturen von Citromyces glaber auf Zucker. *Ibid.* **131**: 338-350. *f. 3.* 1922.

Chace, E. M., Wilson, C. P., and Church, C. G. ('21). The composition of California lemons. U. S. Dept. Agr., Bur. Chem. Bul. 993: 1-18. *f. 1-4.* 1921.

Clark, W. M. ('20). The determination of hydrogen ions. 317 pp. *38 f.* 1920.

Colby, E. ('92). Analyses of California oranges and lemons. Calif. Agr. Exp. Sta., Rept. **1892-94**: 240-256. 1894.

Collison, S. C. ('13). Sugar and acid in oranges and grapefruit. Univ. Fla. Agr. Exp. Sta., Bul. 115: 1-23. 1913.

Creuse, J. ('72). On the estimation of citric acid, free and combined, and a new series of compound citrates. Chem. News **26**: 50-52. 1872.

Currie, J. N. ('17). The citric acid fermentation of Aspergillus niger. Jour. Biol. Chem. **31**: 15-37. 1917.

————, and Thom, C. ('15). An oxalic acid producing Penicillium. *Ibid.* **22**: 287-293. 1915.

Davis, C. E., Oakes, E. T., and Salisbury, H. M. ('23). Titration curves for some common acids and bases as determined by the hydrogen electrode. Jour. Ind. and Eng. Chem. **15**: 182-185. *f. 1.* 1923.

Denigés, G. ('98). Sur les fonctions organiques pouvant se combiner au sulfate mercurique. Cas des acetones. Compt. Rend. Acad. Paris **126**: 1868-1871. 1898.

————. ('98a). Combinaison, recherche et dosage de l'acetone ordinaire avec le sulfate mercurique. *Ibid.* **127**: 963-965. 1898.

Fawcett, H. S. ('12). The cause of stem-end rot of citrus fruits (Phomopsis citri). Phytopath. **2**: 109–113. *pl. 8–9.* 1912.

————. ('15). Citrus diseases of Florida and Cuba compared with those of California. Univ. Calif. Coll. Agr. Exp. Sta., Bul. 262: 153–210. *f. 1–24.* 1915.

Fitz, A. ('78). Ueber Spaltpilzgahrungen. Ber. d. deut. chem. Ges. **11**: 1890–1899. 1878.

Fleisher, A. ('72). Ueber die Einwirkung des ubermangansäuren Kalis auf Weinsäure. *Ibid.* **5**: 350–353. 1872.

Friedeman, T. E. ('21). The determination of carbon in soils and agricultural products. Thesis, Univ. Mo. 126 pp. *6 f.* 1921.

Gadais, L. et J. ('09). Nouvelle methode d'analyse des citrates de chaux et jus de citrons. Soc. Bot. Fr., Bul. IV. **5**: 287–289. 1909.

Gray, G. P., and Ryan, H. J. ('21). Reduced acidity in oranges caused by certain sprays. Calif. Dept. Agr., Month. Bul. **10**: 11–33. 1921.

Haas, A. R. ('17). The reaction of plant protoplasm. Bot. Gaz. **63**: 232–235. 1917.

Hawkins, L. A. ('21). A physiological study of grapefruit ripening and storage. Jour. Agr. Res. **22**: 263–279. 1921.

Heide, C. von der, und Steiner, H. ('09). Über die Bestimmung der Bernsteinsäure im Weine. Zeitschr. f. Untersuch. d. Nahr.- u. Genussmittel **17**: 291–315. 1909.

Hemmi, T. ('20). Beiträge zur Kenntnis der Morphologie und Physiologie der japanischen Gleosporien. Hokkaido Imp. Univ. Coll. Agr. Jour. **9**: 1–59. *pl. 1–3.* 1920.

Jörgensen, G. ('07). Über die Bestimmung einiger der in den Pflanzen vorkommenden organischen Säuren. Zeitschr. f. Untersuch. d. Nahr.- u. Genussmittel **13**: 241–257. 1907.

————. ('09). Über die Bestimmung einiger organischen Pflanzensäuren. *Ibid.* **17**: 396–412. 1909.

Kunz, R. ('14). Occurrence and determination of citric acid in wine, milk, marmalade and fruit sirups. Arch. Chem. Mikros. **7**: 285–299. 1914. Abstracted in Chem. Abstr. **9**: 687–688. 1915.

Leake, C. D. ('23). The occurrence of citric acid in sweat. Am. Jour. Physiol. **63**: 540–544. 1923.

Leffmann, H. ('17). Acid derivatives of alcohols. In Allen's Commercial organic analysis **1**: 485–570. 1917.

Maasen, D. A. ('95). Beiträge zur Ernahrungsphysiologie der Spaltpilz. Die organischen Säuren als Nahrstoffe und ihre Zersetzbarkeit durch die Bakterien. Arb. a. d. K. Gesund. **12**: 340–411. 1895.

McClure, W. B., and Sauer, L. W. ('22). Comparison of pentabromacetone method and Salant and Wise's method for citric acid determination in urine. Am. Jour. Physiol. **62**: 140–144. 1922.

Marriott, W. M. ('13). The determination of acetone. Jour. Biol. Chem. **16**: 281–288. 1913.

————. ('13a). The determination of β-oxybutyric acid in blood and tissues. *Ibid.* **16**: 293–298. 1913.

Martin, J. A. ('16). Citric acid by fermentation. Am. Jour. Pharm. **88**: 337–355. 1916.

Maze, P. ('09). Note sur la production d'acide citrique par les Citromyces (Wehmer). Inst. Pasteur Ann. **23**: 830–833. 1909.

Molliard, M. ('22). Sur une nouvelle fermentation acide produite par le Sterigmatocystis nigra. Compt. Rend. Acad. Paris **174**: 881–883. 1922.

Mulliken, S. P. ('04). A method for the identification of pure organic compounds. **1**. Compounds of order I. 264 pp. *8 f.* 1904.

Nägeli, C. von. ('80). Ernährung der niederen Pilz durch Kohlenstoff und Stickstoff verbindungen. K. b. Akad. d. Wiss. Munchen, Sitzungsber. **10**: 277–367. 1880.

Oppenheimer, C. ('99). Ueber die quantitative Fallung von Aceton mit Quecksilberoxydsulfat. Ber. d. deut. chem. Ges. **32**: 986–988. 1899.

Patterson, W. W., Charles, V. K., Veihmeyer, F. F. ('10). Some fungous diseases of economic importance. Part I. Miscellaneous diseases. U. S. Dept. Agr. Bur. Pl. Ind. Bul. 171: 1–14. 1910.

Pierce, N. B. ('02). Black rot of oranges. Bot. Gaz. **33**: 234–235. 1902.

Pratt, D. S. ('12). Determination of citric acid. U. S. Dept. Agr. Bur. Chem. Circ. 88: 1–7. 1912.

Reed, G. B. ('14). The oxidases of acid tissues. Bot. Gaz. **57**: 528–530. 1914.

Salant, W., and Wise, L. E. ('16). The action of sodium citrate and its decomposition in the body. Jour. Biol. Chem. **28**: 27–58. 1916.

Sando, C. E., and Bartlett, H. H. ('21). Notes on the organic acids of Pyrus coronaria, Rhus glabra and Acer saccharum. Jour. Agr. Res. **22**: 221–229. 1921.

Schollenberger, C. J. ('16). Total carbon in soils by wet combustion. Jour. Ind. and Eng. Chem. **8**: 1126. 1916.

Shaffer, P. A. ('08). A method for the quantitative determination of β-oxybutyric acid in urine. Jour. Biol. Chem. **5**: 211–223. 1908.

————. and Hartmann, A. F. ('21). The iodometric determination of copper and its use in sugar analysis. II. Methods for the determination of reducing sugars in blood, urine, milk, and other solutions. *Ibid.* **45**: 365–390. 1921.

————. and Marriott, W. McK. ('13). The determination of oxybutyric acid. *Ibid.* **16**: 265–280. 1913.

Spindler, O. von. ('03). Zitronensäurebestimmung Mittels der Kalkmethode. Chem. Zeit. **27**: 1263–1264. 1903.

Thiele, A. ('11). Vier- und mehrwertige, dreibasische Säuren (Oxytricarbonsäuren). Abderhalden's biochem. Handlexikon **1**: 1174–1182. 1911.

Thom, C. ('10). Cultural studies of species of Penicillium. U. S. Dept. Agr., Bur. An. Ind. Bul. 118: 1–101. *f. 1–36.* 1910.

————. and Currie, J. N. ('16). The Aspergillus niger group. Jour. Agr. Res. **7**: 1–15. 1916.

Truog, ('15). Methods for the determination of carbon dioxide and a new form of absorption tower adapted to the titrimetric method. Jour. Ind. and Eng. Chem. **7**: 1045–1049. *f. 1.* 1915

Waterman, H. I. ('13). Beitrag zur Kenntniss der Kohlenstoffnahrung von Aspergillus niger. Folia Microbiol. **1**: 422–485. 1913.

Webb, R. W. ('21). Studies in the physiology of the fungi. XV. Germination of the spores of certain fungi in relation to hydrogen-ion concentration. Ann. Mo. Bot. Gard. **8**: 283–341. *f. 1–39.* 1921.

Wehmer, C. ('93). Beiträge zur Kenntniss einheimischer Pilze. I. Zwei neue Schimmelpilze als Erreger einer Citronensäure-Gahrung. *2 pl.* Hanover, 1893.

Willaman, J. J. ('16). Modification of the Pratt method for the determination of citric acid. Am. Chem. Soc., Jour. **38**: 2193–2199. 1916.

Wolf, C. G. L. ('22). The mechanism of the reversal in reaction of a medium which takes place during growth of B. diphtheriae. Biochem. Jour. **16**: 541–547. 1922.

Wolf, E. A., and Shunk, I. V. ('21). Tolerance to acid of certain bacterial plant pathogens. Phytopath **11**: 244–250. 1921.

Wohlk, A. ('02). Über die Einwirkung von Brom und Kalium permanganat auf Citronensäure (Stahre's Reaction) und den Nachweis von Citronensäure in Milch. Zeitschr. f. analyt. Chem. **41**: 77–100. 1902.

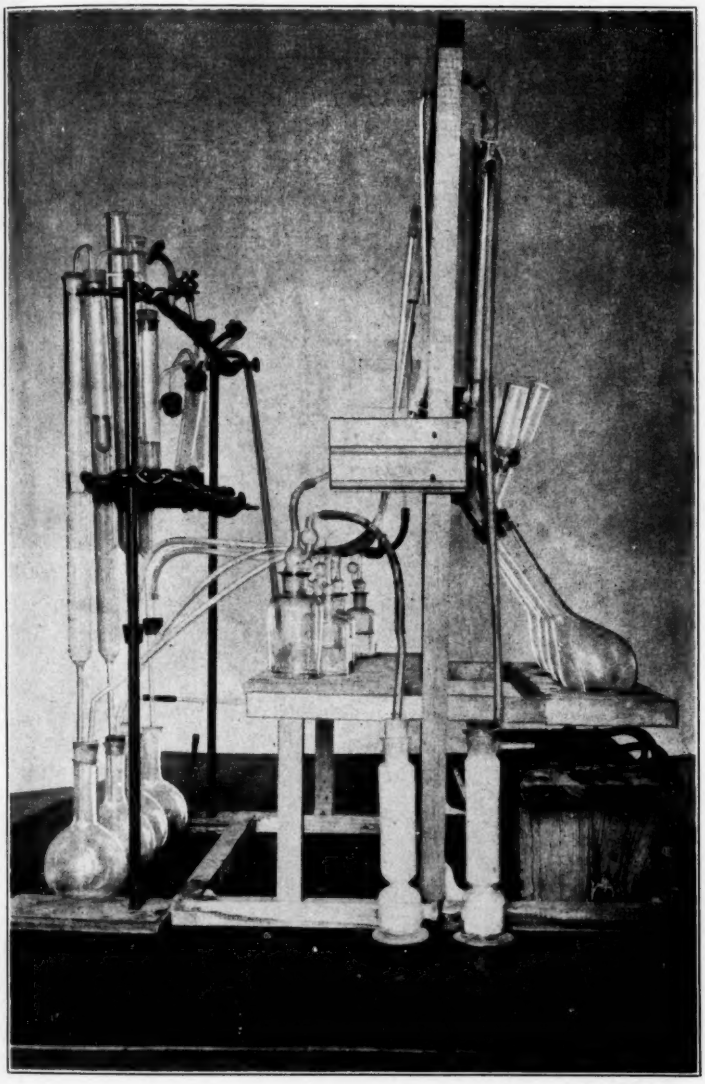
Yoder, P. A. ('11). Notes on the determination of acid in sugar cane juice. Jour. Ind. and Eng. Chem. **3**: 640–646. 1911.

————. ('11a). A polariscopic method for the determination of malic acid and its application in cane and maple products. *Ibid.* **3**: 563–574. 1911.

Explanation of Plate

PLATE 14

Carbon analysis equipment (see page 237).



CAMP—CITRIC ACID AS A SOURCE OF CARBON